PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 5/10, A01H 5/00

A1

(11) International Publication Number: **WO 97/06269**

(43) International Publication Date: 20 February 1997 (20.02.97)

(21) International Application Number: PCT/GB96/01883

(22) International Filing Date: 2 August 1996 (02.08.96)

(30) Priority Data:
9515941.4 3 August 1995 (03.08.95) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: JEPSON, Ian [GB/GB]; 31 Gringer Hill, Maidenhead, Berkshire SL6 7LY (GB).

(74) Agents: BISHOP, Nigel, Douglas et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INDUCIBLE HERBICIDE RESISTANCE

(57) Abstract

The invention relates to DNA constructs which are capable of conferring on a plant inducible resistance to a herbicide. The inducible effect may be achieved by using a gene switch such as the *alcA/alcR* switch derived from *A. nidulans*. The invention relates in particular to inducible resistance to the herbicide N-phosphonomethyl glycine (glyphosate) and its salts.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

INDUCIBLE HERBICIDE RESISTANCE

The present invention relates to DNA constructs and plants incorporating them. In particular it relates to promoter sequences for the expression of genes which confer herbicide resistance on plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from Streptomyces hydroscopicus. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from Arabidopsis, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter.

We propose a system where genes conferring herbicide tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide resistance genes. This may be a particular problem as early stages of growth where high levels of transgene product may directly interfere with normal development. Alternatively high levels of expression of herbicide resistance genes may cause a metabolic drain for plant resources.
2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
3. The use of an inducible promoter to drive herbicide resistance genes will reduce the risk of resistant weed species becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide

resistance in cereals, such as wheat, might outcross into the weed wild oats or that herbicide resistance in oil seed rape or canola could be transferred to wild brassicas thus conferring herbicide resistance to these already troublesome weeds. A further example is that the inducible expression of herbicide resistance in sugar beet will reduce the risk of wild sugar beet becoming a problem.

Several gene regulation systems (gene switches) are known and may be used for conferring inducible herbicide resistance on plants. Many such gene switches are described in the review by Gatz (Current Opinion in Biotechnology (1996) 7, 168-172) and include systems such as the tetracycline repressor gene switch, the Lac repressor system, copper inducible systems such as that based on ACE 1, salicylic acid inducible promoters including the PR-1a system and systems based on sterioid hormones such as the glucocorticoid, progesterone and oestrogen receptor systems. Modifications of the glucocorticoid receptor systems which include the GAL 4 binding domain from yeast and the VP16 activator are described by Aoyama *et al, The Plant Cell*, (1995) 7, 1773-1785 and it is envisaged that similar systems may based on, for example insect steroid hormones rather than on mammalian steriod hormones. Indeed, a system based on the ecdysone receptor of *Heliothis virescens* has recently been described. Benzene sulphonamide gene switching systems are also known (Hershey *et al*, *Plant Mol. Biol.*, 17, 679-690 (1991) as are systems based on the alcR protein from *Aspergillus nidulans* and glutathione S-transferase promoters.

Several genes which confer herbicide resistance are also known, for example, one herbicide to which resistance genes have been described and which is extremely widely used is N-phosphonomethyl-glycine (glyphosate) and its agriculturally acceptable salts including the isopropylamine, trimethylsulphonium, sodium, potassium and ammonium salts.

In a first aspect of the present invention there is provided a chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.

Any herbicide resistance gene may be used but genes which confer resistance to N-phosphonomethyl-glycine or salts or derivatives thereof are especially preferred.

Several inducible promoters may be used to confer the inducible resistance and these include any of those listed above.

However, a particularly useful gene switch for use in this area is based on the *alc* R regulatory protein from *Aspergillus nidulans* which activates genes expression from the *alc*A promoter in the presence of certain alcohols and ketones. This system is described in our International Patent Publication No. WO93/21334 which is incorporated herein by reference.

The *alc*A/*alc*R gene activation system from the fungus *Aspergillus nidulans* is also well characterised. The ethanol utilisation pathway in *A. nidulans* is responsible for the degradation of alcohols and aldehydes. Three genes have been shown to be involved in the ethanol utilisation pathway. Genes *alc*A and *alc*R have been shown to lie close together on linkage group VII and *ald*A maps to linkage group VIII (Pateman JH *et al*, 1984, Proc. Soc. Lond, B217:243-264; Sealy-Lewis HM and Lockington RA, 1984, Curr. Genet, 8:253-259). Gene *alc*A encodes ADHI in *A. nidulans* and *ald*A encodes AldDH, the second enzyme responsible for ethanol utilisation. The expression of both *alc*A and *ald*A are induced by ethanol and a number of other inducers (Creaser EH *et al*, 1984, Biochemical J, 255:449-454) via the transcription activator *alc*R. The *alc*R gene and a co-inducer are responsible for the expression of *alc*A and *ald*A since a number of mutations and deletions in *alc*R result in the pleiotropic loss of ADHI and aldDH (Felenbok B *et al*, 1988, Gene, 73:385-396; Pateman *et al*, 1984; Sealy-Lewis & Lockington, 1984). The ALCR protein activates expression from *alc*A by binding to three specific sites in the *alc*A promoter (Kulmberg P *et al*, 1992, J. Biol. Chem, 267:21146-21153).

The *alc*R gene was cloned (Lockington RA *et al*, 1985, Gene, 33:137-149) and sequenced (Felenbok *et al*, 1988). The expression of the *alc*R gene is inducible, autoregulated and subject to glucose repression mediated by the CREA repressor (Bailey C and Arst HN, 1975, Eur. J. Biochem, 51:573-577; Lockington RA *et al*, 1987, Mol. Microbiology, 1:275-281; Dowzer CEA and Kelly JM, 1989, Curr. Genet, 15:457-459; Dowzer CEA and Kelly JM, 1991, Mol. Cell. Biol, 11:5701-5709). The ALCR regulatory protein contains 6 cysteines near its N terminus co-ordinated in a zinc binuclear cluster (Kulmberg P *et al*, 1991, FEBS Letts, 280:11-16). This cluster is related to highly conserved DNA binding domains found in transcription factors of other ascomycetes. Transcription factors GAL4 and LAC9 have been shown to have binuclear complexes which have a cloverleaf type structure containing two Zn(II) atoms (Pan T and Coleman JE, 1990, Biochemistry, 29:3023-3029; Halvorsen YDC *et al*, 1990, J. Biol. Chem, 265:13283-13289). The structure of ALCR is

similar to this type except for the presence of an asymmetrical loop of 16 residues between Cys-3 and Cys-4. ALCR positively activates expression of itself by binding to two specific sites in its promoter region (Kulmberg P *et al*, 1992, Molec. Cell. Biol, 12:1932-1939).

The regulation of the three genes, *alc*R, *alc*A and *ald*A, involved in the ethanol utilisation pathway is at the level of transcription (Lockington *et al*, 1987; Gwynne D *et al*, 1987, Gene, 51:205-216; Pickett *et al*, 1987, Gene, 51:217-226).

There are two other alcohol dehydrogenases present in *A. nidulans*. ADHII is present in mycelia grown in non-induced media and is repressible by the presence of ethanol. ADHII is encoded by *alc*B and is also under the control of *alc*R (Sealy-Lewis & Lockington, 1984). A third alcohol dehydrogenase has also been cloned by complementation with a *adh*- strain of *S. cerevisiae*. This gene *alc*C, maps to linkage group VII but is unlinked to *alc*A and *alc*R. The gene, *alc*C, encodes ADHIII and utilises ethanol extremely weakly (McKnight GL *et al*, 1985, EMBO J, 4:2094-2099). ADHIII has been shown to be involved in the survival of *A. nidulans* during periods of anaerobic stress. The expression of *alc*C is not repressed by the presence of glucose, suggesting that it may not be under the control of *alc*R (Roland LJ and Stromer JN, 1986, Mol. Cell. Biol, 6:3368-3372).

In summary, *A. nidulans* expresses the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene *alc*A only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alc*R gene and constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein *activates* the expression of the *alc*A gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADH1 enzyme are produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the *alc*A gene and its product, ADH1, are not expressed in the absence of inducer. Expression of *alc*A and production of the enzyme is also repressed in the presence of glucose.

Thus the *alc*A gene promoter is an inducible promoter, activated by the *alc*R regulator protein in the presence of inducer (i.e. by the protein/alcohol or protein/ketone combination). The *alc*R and *alc*A genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al*, 1985, Gene, 33:137-149; Felenbok B *et al*, 1988, Gene, 73:385-396; Gwynne *et al*, 1987, Gene, 51:205-216).

Alcohol dehydrogenase (*adh*) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of *adh* genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the *alc*R regulator protein has been found in any plant. Hence the *alc*R/*alc*A type of gene regulator system is not known in plants. Constitutive expression of *alc*R in plant cells does not result in the activation of endogenous *adh* activity.

According to a second aspect of the invention, there is provided a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to an *alc*R regulator sequence which encodes an *alc*R regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

The inducible promoter is preferably derived from the *alc*A gene promoter but may, alternatively be derived from *alc*R, *ald*A or other *alc*R-induced genes.

We have found that the *alc*A /*alc*R switch is particularly suited to drive herbicide tolerance genes for at least the following reasons.

1. The *alc*A/*alc*R switch has been developed to drive high levels of gene expression. In addition, the regulatory protein *alc*R is preferably driven from a strong constitutive promoter such as polyubiquitin. High levels of induced transgene expression, comparable to that from a strong constitutive promoter, such as 35 CaMV, can be achieved.

2. If a gene switch is to be used in a situation where the activating chemical is applied simultaneously with the herbicide, a rapid elevation in the levels of herbicide resistance gene is required. Figure 1 reveals a time course of marker gene expression (CAT) following application of inducing chemical. This study shows a rapid increase (2 hours) of CAT expression following foliar application of inducing chemical. The immediate early kinetics of induction are brought about be expressing the regulatory protein in constitutive manner, therefore no time lag is encountered while synthesis of transcription factors takes place. In addition we have chosen a simple two component system which does not rely on a complex signal transduction system.

3. We have tested the specificity of *alcA/alcR* system with a range of solvents used in agronomic practice. A hydroponic seedling system revealed that ethanol, butan-2-ol and cyclohexanone all gave high levels of induced reporter gene expression (Figure 2). In contrast when the alcohols and ketones listed in Table 1 in which are used in agronomic practice were applied as a foliar spray only ethanol gave high levels of induced reporter gene activity (Figure 3).

Table 1

1.	Isobutyl methyl ketone	13.	acetonyl acetone
2.	Fenchone	14.	JF5969 (cyclohexanone)
3.	2-heptanone	15.	N-methyl pyrrolidone
4.	Di-isobutyl ketone	16.	polyethylene glycol
5.	5-methyl-2-hexanone	17.	propylene glycol
6.	5-methylpentan-2,4-diol	18.	acetophenone
7.	ethyl methyl ketone	19.	JF4400 (methylcyclohexanone)
8.	2-pentanone	20.	propan-2-ol
9.	glycerol	21.	butan-2-ol
10.	γ-butyrolactone	22.	acetone
11.	diacetone alcohol	23.	ethanol
12.	tetrahydrofurfuryl alcohol	24.	dH_2O

This is of significance since illegitimate induction of transgenes will not be encountered by chance exposure to formulation solvents. Ethanol is not a common component of agrochemical formulations and therefore with appropriate spray management can be considered as a specific inducer of the *alc* A / R gene switch in a field situation.

4. A range of biotic and abiotic stresses for example pathogen infection, heat, cold, drought, wounding, flooding have all failed to induce the *alcA /alcR* switch. In addition a range of non-solvent chemical treatments for example salicylic acid, ethylene, absisic acid, auxin, gibberelic acid, various agrochemicals, all failed to induce the *alcA / alcR* system.

The first promoter may be constitutive or tissue-specific, developmentally-programmed or even inducible. The regulator sequence, the *alc*R gene, is obtainable from *Aspergillus nidulans*, and encodes the *alc*R regulator protein.

The inducible promoter is preferably the *alc*A gene promoter obtainable from *Aspergillus nidulans* or a "chimeric" promoter derived from the regulatory sequences of the *alc*A promoter and the core promoter region from a gene promoter which operates in plant cells (including any plant gene promoter). The *alc*A promoter or a related "chimeric" promoter is activated by the *alc*R regulator protein when an alcohol or ketone inducer is applied.

The inducible promoter may also be derived from the *ald*A gene promoter, the *alc*B gene promoter or the *alc*C gene promoter obtainable from *Aspergillus nidulans*.

The inducer may be any effective chemical (such as an alcohol or ketone). Suitable chemicals for use with an *alc*A/*alc*R-derived cassette include those listed by Creaser *et al* (1984, Biochem J, 225, 449-454) such as butan-2-one (ethyl methyl ketone), cylcohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to an applied exogenous chemical inducer enabling external activation of expression of the target gene regulated by the cassette. The expression cassette is highly regulated and suitable for general use in plants.

The two parts of the expression cassette may be on the same construct or on separate constructs. The first part comprises the regulator cDNA or gene sequence subcloned into an expression vector with a plant-operative promoter driving its expression. The second part comprises at least part of an inducible promoter which controls expression of a downstream target gene. In the presence of a suitable inducer, the regulator protein produced by the first part of the cassette will activate the expression of the target gene by stimulating the inducible promoter in the second part of the cassette.

In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti

plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

The invention further provides a method for controlling plant gene expression comprising transforming a plant cell with a chemically-inducible plant gene expression cassette which has a first promoter operatively linked to an *alc*R regulator sequence which encodes an *alc*A regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide.

This strategy can be adopted for any resistance conferring gene/corresponding herbicide combination. For example , the *alc*A/*alc*R gene switch can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.

3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No WO90/14000) and other genes, which confer resistance to sulphonyl urea and imadazlonones.

4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377 in the name of Monsanto Company); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201 in the name of Monsanto Company); and genes which are involved in the expression of CPLyase,

Various further preferred features and embodiments of the present invention will now be described in the non-limiting examples set out below and with reference to the drawings in which:

Figure 1 illustrates the time course of marker gene expression (CAT) following application of inducing chemical;

Figure 2 illustrates the levels of induced reporter gene expression on root drenching with a range of solvents;

Figure 3 illustrates the levels of induced reporter gene activity when the chemicals listed in Table 1 were applied as a foliar spray;

Figure 4 illustrates the production of the 35S regulator construct by ligation of *alc*R cDNA into pJR1.

Figure 5 illustrates the production of the reporter construct;

Figure 6 is a summary of the cassettes and specific plant transformation constructs;

Figure 7 illustrates the chloroplast transit sequence 1 from Arabidopsis RUBISCO (CPT 1);

Figure 8 shows the sequence of plasmid pMJB1;

Figure 9 is a map of plasmid pJRIi;

Figure 10 illustrates the chloroplast transit sequence CTP2 from EPSPS class I gene from Petunia hybrida;

Figure 11 is a map of plasmid pUB-1;

Figure 12 is a map of plasmid pMF6;

Figure 13 is a map of plasmid pIE109 in which the numbers are in base pairs (not to scale) and the following abbreviations are used:

ADH_i	Alcohol dehydrogenase from maize;
PAT	Phosphinothricin acetyl transferase (Basta resistance gene);
AMP	Ampicillin resistance gene;
CaMV 35S	Cauliflower mosaic virus 35S promoter;
nos Poly A	Nopaline synthase poly A region;
ori	ColE1 origin of replication from pUC

Figure 14 is a map of plasmid pMV1 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 13 with the following additional abbreviations:

UBQ_p	Maize ubiquitin promoter;
UBQ_i	Maize ubiquitin intron;
nos	Nopaline synthase 3' terminator;
CZP1 GOX	Chloroplast transit peptide - glyphosate oxidase sequence;
CZP2 GPSPS	Chloroplast transit peptide - EPSP synthetase sequence;

Figure 15 shows the preparationof plasmid pUC4 by ligation of pAr3 and pBSSK;

Figure 16 is a map of plasmid pMV2 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 14 with the following additional abbreviations:

*Alc*A	*Aspergillus nidulans alc*A promoter;
*Alc*R	*Aspergillus nidulans alc*R promoter;

Figure 17 is a map of plasmid pDV1-pUC;

Figure 18 is a map of plasmid pDV2-pUC;

Figure 19 is a map of plasmid pDV3-Bin;

Figure 20 is a map of plasmid pDV4-Bin; and

Figure 21 is a western Blot showing the expression of EPSPS and GOX in transformants.

EXAMPLES

We have chosen to exemplify the *alc*A/*alc*R gene switch with genes conferring resistance to glyphosate. The switch will be used to drive inducible expression of glyphosate oxidase (GOX) in plants. Switchable GOX has been expressed alone or in conjunction with constitutive expression of 5-enol-pyruvylshikimate 3-phosphate (EPSPS) CP4. Constructs have been optimised for expression in monocotyledonous and dicotyledonous crop species.

EXAMPLE 1

Production Of The *alc*R Regulator Construct.

The *alc*R genomic DNA sequence has been published, enabling isolation of a sample of *alc*R cDNA.

The *alc*R cDNA was cloned into the expression vectors pJR1(pUC). pJR1 contains the Cauliflower Mosaic Virus 35S promoter. This promoter is a constitutive plant promoter and will continually express the regulator protein. The *nos* polyadenylation signal is in the expression vector.

Figure 4 illustrates the production of the 35S regulator construct by ligation of *alc*R cDNA into pJR1. Partial restriction of the *alc*R cDNA clone with *Bam*HI was followed by electrophoresis in an agarose gel and the excision and purification of a 2.6 Kb fragment. The fragment was then ligated into the pJR1 vector which had been restricted with *Bam*HI and phosphatased to prevent recircularisation. The *alc*R gene was thus placed under control of the CaMV 35S promoter and the *nos* 3' polyadenylation signal in this "35S-*alc*R" construct.

EXAMPLE 2

Production Of The *alc*A-CAT Reporter Construct Containing The Chimeric Promoter.

The plasmid pCaMVCN contains the bacterial chloramphenicol transferase (CAT) reporter gene between the 35S promoter and the *nos* transcription terminator (the "35S-CAT" construct).

The *alc*A promoter was subcloned into the vector pCaMVCN to produce an "*alc*A-CAT" construct. Fusion of part of the *alc*A promoter and part of the 35S promoter created a chimeric promoter which allows expression of genes under its control.

Figure 5 illustrates the production of the reporter construct. The *alc*A promoter and the 35S promoter have identical TATA boxes which were used to link the two promoters together using a recombinant PCR technique: a 246 bp region from the *alc*A promoter and

the 5' end of the CAT gene from pCaMVCN (containing part of the -70 core region of the 35S promoter) were separately amplified and then spliced together using PCR. The recombinant fragment was then restriction digested with *Bam*HI and *Hin*dIII. The pCaMVCN vector was partially digested with *Bam*HI and *Hin*dIII, then electrophoresed so that the correct fragment could be isolated and ligated to the recombinant fragment.

The ligation mixtures were transformed into *E coli* and plated onto rich agar media. Plasmid DNA was isolated by miniprep from the resultant colonies and recombinant clones were recovered by size electrophoresis and restriction mapping. The ligation junctions were sequenced to check that the correct recombinants had been recovered.

EXAMPLE 3

Glyphosate Resistance Constructs

A summary of the cassettes and specific plant transformation constructs is shown in Figure 6.

Dicot Vector 1

Vector 1 is a constitutive control plasmid containing the glyphosate oxidase gene (GOX) fused to the chloroplast transit sequence 1 from Arabidopsis RUBISCO (CPT 1) (Figure 7) driven by the enhanced 35S CaMV promoter (ES) and the TMV omega translational enhancer sequence (TMV). Vector 1 utilizes the nopoline synthase terminator (nos). The synthetic GOX gene with the addition of CTP 1 was synthesised with information from patent publication WO92/00377 with addition of *Nco*I site at the translation start ATG, and a *Kpn* I at the 5' end. Internal *Sph* I sites and *Nco*I site were deleted during synthesis with no change in amino acid usage. The CTP 1 GOX synthesised sequence was isolated as a *Nco* I *Kpn* I fragment and ligated using standard molecular cloning techniques into *Nco*I *Kpn*I cut pMJB1, a plasmid based on pIBT 211 containing the CaMV 35 promoter with duplicated enhancer linked to the tobacco mosaic virus translational enhancer sequence replacing the tobacco etch virus 5' non-translated leader, and terminated with the nopaline synthase poly (A) signal (nos) (Figure 8).

A cassette containing enhanced 35 CaMV TMV sequence CTP1 GOX and nos terminator (dicot vector 1 pUC Figure 17) was isolated as a *Hin*dIII *Eco*RI fragment and ligated into *Hin*d III *Eco*RI cut pJRIi, a Bin 19 base plant transformation vector (Figure 9).

Dicot Vector 2

The synthetic EPSPS CP4 gene, fused to the chloroplast transit sequence CTP2 (Figure 10) from EPSPS class I gene from Petunia hybrida, was synthesised with data from patent WO 92/04449 with *Nco*I at the translation initiation ATG. A internal *Sph* I site was silenced in the EPSPS CP4 gene with no change of amino acid usage.

A fragment containing the synthetic CTP 2 CP4 EPSPS was isolated as a *Nco*I *Sac* I fragment and ligated in to pMJBI. A fragment containing the CaMV 35 promoter with a duplicated enhancer, TMV omega sequence CTP 2 transit peptide, EPSPS and nos terminator was isolated as a *Eco*RI *Hin*d III fragment (dicot vector 2 pUC Figure 18)and cloned into pJRIi to give dicot vector 2 pUC (Figure 18).

Upon sequencing the junctions of dicot vector 2, an additional sequence was identified inserted between the *Sac*I site and the beginning of the nos terminator. This was as follows:

5′ AGG CTG CTT GAT GAG CTC GGT ACC CGG GGA TCC ATG GAG CCG AAT 3′

Dicot Vector 3

A control vector with both EPSPS and GOX genes was constructed by cutting dicot vector 2 with *Eco*RI and inserting an Δ*Eco*RI *Sph* I Δ*Eco*RI linker. The sequence of the linker is shown below:

5′ AAT TAG GGG CAT GCC CCT 3′

The resultant vector was cut with Sph I to liberate the cassette B which was cloned into an *Sph*I site in dicot vector 1), 5' to the 35 CaMV promoter. Cassettes 1) and 2) were then excised as a *Hin*dIII and *Eco*RI fragment from dicot vector 3- pUC (Figure 19) and cloned in to pJRIi.

Dicot Vector 4

An inducible GOX vector was constructed by excising the CAT gene from "p*alc*CAT" as *Pst*I fragment. The vector band, containing the *alc*A promoter and nos terminator was gel purified and used in ligations with a *Pst*I-*Xho*I-*Kpn*I-*Pst*I linker, the sequence of which is as follows:

5′ GCC ACT CGA GCT AGG TAC CCT GCA 3′

The orientation of this was confirmed by sequence analysis. The TMV omega and CTPI GOX sequence from dicot vector 1) were isolated as a *Xho*I *Kpn*I fragment and cloned into the *alc*A nos vector containing the *Xho*I-*Kpn*I-*Pst*I linker. The *alc*A TMV CTP1 GOX

nos cassette was excised as a *Hin*dIII fragment and cloned into the plant transformation vector "p35S-*alc* R", containing the *alc*R cDNA nos terminator under the control of the 35 CaMV promoter to form dicot vector 4 (Figure 20).

Dicot Vector 5

Dicot vector 5 (Figure 22) containing inducible GOX and constitutive EPSPS genes was prepared using the following cloning strategy. Dicot vector 2 (pDV2 -pUC) was modified by cloning in a Δ*Eco*RI-*Hin*dIII-Δ*Eco*RI linker into the *Eco*RI site to allow excision of the CaMV en-CTP2-EPSPS -nos cassette as a *Hin*dIII fragment. This fragment was then ligated into *Hin*dIII cut pDV4-Bin. Recombinants containing all three cassettes ie 35S-*Alc*R, CaMVen-CTP2-EPSPS-nos and AlcA-CTP1-GOX-nos were selected by hybridization with radiolabelled oligonucleotides. Confirmation of orientation was done by sequencing across all borders.

Monocot Vectors

Vector 1: Cassette D

An *Eco*RI-*Not*I-*Eco*RI linker (5'AATTCATTTGCGGCCGCAAATG3') was inserted into dicot vector pDV1. The plasmid was cut with *Nco*I and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with *Not*I and the resulting blunt/*Not*I fragment containing the CTP1 GOX and nos terminator was ligated into a *Sma*I/*Not*I digested pPUB1 vector (Figure 12) containing the polyubiquitin promoter, polyubiquitin intron with a *Kpn*I-*Not*I-*Kpn*I linker (5'CATTTGCGGCCGC AAATGGTAC3') insertion. A *Hin*dIII-*Not*I-*Hin*dIII linker (5'AGCTTGCAGCGGC CGCTGCA3') was inserted into the resulting construct.

Vector 1: Cassette E

An *Eco*RI-*Not*I-*Eco*RI linker (5'AATTCATTTGCGGCCGCAAATG3') was inserted into dicot vector pDV2. The plasmid was cut with *Nco*I and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with *Not*I and the resulting blunt/*Not*I fragment containing the CTP2 EPSPS and nos terminator was ligated into a *Sma*I/*Not*I digested pPUB1 vector containing the polyubiquitin promoter, polyubiquitin intron with a *Kpn*I-*Not*I-*Kpn*I linker (5'CATTTGCGGCCGCAAATGGT AC3') insertion to create plasmid 1. The PAT selectable marker cassette (35S CaMV promoter, *Adh*I intron,

phosphinothricin acetyl transferase gene (PAT), nos terminator) was excised from pIE108 (Figure 14) and cloned into the *Hin*dIII site on plasmid 1 to give mononcot cassette E. Diagnostic restriction digestion was used to confirm that the selectable marker cassette was inserted 5' to 3' in the same orientation as the CTP2 EPSPS cassette.

A fragment containing the polyubiquitin promoter, polyubiquitin intron, CTP1 GOX and nos terminator was excised from cassette D with *Not*I and ligated into *Not*I cassette E to form monocot vector 1 (Figure 14). Restriction digestion was used to confirm that the two cassettes were inserted in the same orientation.

The selectable marker cassette (35 CaMV promoter, *Adh*I intron, phosphinothricin acetyl transferase gene (PAT), nos) was excised from pIE108 and cloned into the *Hin*d III site in 5) to give monocot cassette E.

Vector 1

A fragment containing the polyubiquitin promoter, polyubiquitin intron GOX and nos was exised from cassette D with *Not*I and cloned into *Not*I cut casette E, to form monocot vector 1.

Vector 2 Cassette F

An *Eco*RI fragment from pUC4 (Figure 15) containing the *alc*R cDNA and nos terminator sequences was blunt end-filled with DNA Polymerase I Klenow fragment, ligated into pUB1 with the *Kpn*I-*Not*I-*Kpn*I linker insertion and orientated by restriction analysis. The PAT selectable marker cassette was inserted in the *Hin*dIII site after excision from pIE108 and orientated by restriction analysis to create vector 1. Plasmid 1 above containing the polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS and nos terminator was cut with *Hin*dIII and a Δ*Hin*dIII-*Not*I-*Hin*dIII linker:

5'AGCTCGCAGCGGCCGCTGCA3'

5'GCGTCGCCGGCGACGTTCGA3'

inserted and orientated by sequencing to create vector 2.

A *Cla*I-*Nco*I-*Cla*I linker (5'CGATGCAGCCATGGCTGCAT3') was inserted into pMF6 (Figure 13) to give vector 3. An *NcoI*/*Kpn*I fragment containing CTP1 GOX was excised from pDV1 and inserted into *NcoI*/*Kpn*I cut vector 3 to create vector 4. A SalI fragment containing the maize AdhI intron, CTP1 GOX was excised from vector 4 and ligated into SalI cut pUC2 containing the alcA promoter and nos terminator and orientated by sequencing to create vector 5. A *Hin*dIII fragment from vector 5 containing the alcA promoter, maize AdhI

intron, CTP1 GOX and nos terminator was ligated into *Hin*dIII cut vector 2 and orientated by restriction digestion. A *Not*I fragment from the resulting construct containing polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS, nos terminator, alcA promoter, maize AdhI intron, CTP1 GOX and nos terminator was ligated into *Not*I cut vector 1 and orientated by restriction analysis to create monocot vector 2 (Figure 16).

EXAMPLE 4

Plant Transformation

Plasmids for dicot transformation were transferred to Agrobacterium tumefaciens LBA4404 using the freeze thaw method described by Holsters *et al* 1978.

Tobacco transformants were produced by the leaf disc method described by Bevan 1984. Shoots were regenerated on a medium containing 100 mg/l kanamycin. After rooting plants were transferred to the glasshouse and grown under 16h light/8h dark conditions.

Oilseed rape (Brassica napus cv westar) transformations were performed using the cotyledon petiole method described by Moloney *et al* 1989. Selection of transformed material was performed on kanamycin (15 mg/l). Rooted shoots were transferred directly to a soil based compost and grown to maturity under controlled glasshouse conditions (16h day 20°C day, 15°C night 60% RH).

Maize transformation was performed using the particle bombardment approach as described by Klein *et al* 1988. Selections were performed on 1 mg/l biolophos.

Sugar beet transformation was performed using the guard cell protoplast procedure see our International Patent Publication No. WO95/10178.

Results showing details of the transgenic plants obtained are shown in Tables 2 and 3 below.

Table 2 - Transformation Details For Tobacco

Vector	Species	Shoots removed	Rooted
pDV1	Tobacco	150	57
pDV2	Tobacco	150	60
pDV3	Tobacco	270	77
pDV4	Tobacco	350	135
pDV5	Tobacco	150	75

Table 3 - Transformation Details in Oil Seed Rape

Vector	Species	Shooting Calli	Rooted
pDV1	OSR	14	shoots from 14
pDV2	OSR	13	shoots from 13
pDV3	OSR	18	shoots from 18
pDV4	OSR	20	shoots from 20
pDV5	OSR	19	shoots from 18

EXAMPLE 5

Transgenic Plant Analysis

Polymerase Chain Reaction (PCR)

Genomic DNA for PCR analysis of transgenic plants was prepared according to the method described by Edwards *et al* 1992. PCR was performed using conditions described by Jepson *et al* , Plant Molecular Biology Reporter, 9(2), 131-138 (1991). Primer sets were designed for each of the introduced cassettes.

The plants were analysed using the following oligonucleotide combinations:-

pDV1	TMV1 + GOX1,	GOX3 + nos1	
pDV2	TMV1 + EPSPS1,	EPSPS3 + nos1	
pDV3	EPSPS3 + GOX1		
pDV4	35S + AlcR1,	AlcA2 + GOX1	
pDV5	35S + AlcR1,	AlcA2 + GOX1,	TMV1 + EPSPS1

Oligonucleotide sequences are given below:-

TMV1	5′ CTCGAGTATTTTTACAACAATTACCAAC
GOX1	5′ AATCAAGGTAACCTTGAATCCA
GOX3	5′ ACCACCAACGGTGTTCTTGCTGTTGA
NOS1	5′ GCATTACATGTTAATTATTACATGCTT
EPSPS1	5′ GTGATACGAGTTTCACCGCTAGCGAGAC
EPSPS3	5′ TACCTTGCGTGGACCAAAGACTCC
35S	5′ GTCAACATGGTGGAGCACG
AlcR1	5′ GTGAGAGTTTATGACTGGAGGCGCATC
AlcA2	5′ GTCCGCACGGAGAGCCACAAACGA

Selection on Glyphosate

Kill Curves for Tobacco var Samsun and Brassica napus var Westar on glyphosate

Both species were tested on a range of glyphosate concentrations by inserting, in the case of tobacco a 5-6mm stem segment carrying a leaf node and in the case of oil seed rape the growing tip plus two leaves into MS medium containing glyphosate at 0, 0.0055, 0.011, 0.0275, 0.055 and 0.01 mM glyphosate isopropylamine salt. The results were scored after two weeks growth as and are given in Table 4 below.

Table 4

Conc	Westar	Tobacco
0	Good stem growth, 4-5 new leaves, roots up to 5cm	As OSR
0.005	No stem growth, 1 new leaf, roots to 1cm	No growth in any organ
0.011	No stem growth, no new leaves, roots~0.5cm	"
0.0275	No stem growth, no new leaves, roots~2mm	"
0.055	No growth in any organ, ends of stem blackened	"
0.01	As for 0.055mM	"

Selection for glyphosate tolerant transformants was performed on glyphosate concentrations of 0.01 and 0.05mM.

Constitutively tolerant plants

Following from the data obtained on wild type plants, pDV1,2 and 3 PCR +ve primary transformants were screened on MS medium containing glyphosate at the levels described above. For tobacco this was done by inserting three or four stem sections per transformant into the medium and using untransformed Samsun as control. Scoring was based on the behaviour of the majority. Plants showing tolerance at the higher concentration of herbicide were grown on to maturity in the glass house, for seed collection.

Segregation Test

Seeds were sterilized in 10% bleach for 10 min. After several washes in sterile water 200 seeds were sown on 1/2 MS medium (2.3 g/l MS salt, 1.5% sucrose, 0.8% Bactoagar,

pH 5.9) containing 100 mg/l kanamycin. Seeds were grown at 26°C with 16 hours/8 hours light/dark prior to scoring.

Western Analysis

Antibody Generation

GOX and EPSPS protein were over expressed in E.Coli using a pET expression system. Following IPTG induction GOX and EPSPS were electro eluted from the shake flask grown cell paste and used to immunise rabbits (two animals per clone).

Preparation of Tissue Extracts for Immunoblotting

120 mg of leaf tissue plus 60 mg PVPP and 500 µl extraction buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 0.3 mM DTT) were ground with a blender for several minutes. After homogenisation the extract was centrifuged at 15,000 rpm for 15 min. The supernatant was stored at -80° C until required. Protein concentrations in the extract were measured according to Bradford.

SDS-PAGE and Immunoblotting

25 µg protein were separated by SDS-PAGE. The running buffer was 14.4 % (w/v) glycine, 1 % (w/v) SDS and 3 % (w/v) Tris Base. The samples were loaded according to Laemmli.

After SDS-PAGE proteins were electroblotted overnight with 40 mA onto nitrocellulose (Hybond™ C, Amersham) using an electroblot unit from Biorad. The membrane was stained in 0.05 % CPTS dissolved in 12 mM HCl. Blots were rinsed in 12 mM HCl and destained for 5 - 10 min in 0.5 M $NaHCO_3$ followed by an intensive rinse with H_2O. Membranes were then blocked, immunodetected and washed according to the Amersham ECL kit. Indirect immunodetections were performed with a 1:10000 dilution of a rabbit anti-GOX or anti-EPSPS polyclonal as first antibody and with a 1:1000 dilution of an anti-rabbit second antibody, associated with horseradish peroxidase. An additional wash was carried out overnight to eliminate background. Detection was performed using the ECL kit from Amersham and the results are shown in Figure 21 in which Lane (1) is the control and the remaining lanes are transformants. The western analysis demonstrates that some transformants are capable of expressing GOX and EPSPS.

Constitutively tolerant plants

Cell extracts were prepared from each glyphosate tolerant plant and the amount of expresssed protein estimated by western analysis using antibody appropriate to the

transformant. Plants expressing very high levels of GOX or EPSPS were tested on higher levels of glyphosate to relate level of expression to herbicide tolerance.

Inducibly tolerant plants

To demonstrate inducible tolerance to glyphosate PCR positive primary transformants from the transformations with pDV4 and 5 were transferred directly to the glass house. After two weeks the plants were induced by an ethanol root drench (5% solution) and left for 24 hours prior to western analysis performed to assess level of expression of GOX after induction. After a period of time to allow the plants to return to the uninduced state, the western analysis was repeated to allow selection of inducibly tolerant plants. Plants which showed the highest levels of GOX expression following ethanol treatment were taken forward to time course analysis. GOX levels were assessed at 6, 12, 18, 24, 36, 48 hours following ethanol treatment, by western analysis.

High expressing GOX plants for both pDV4 and pDV5 were used in glass house trials to demonstrate inducible glyphosate tolerance. Plants were induced using a range of ethanol concentrations (1-15%) by root drench application to pot grown plants. Following GOX induction plants were sprayed with glyphosate. Wild type controls and uninduced plants were also treated with herbicide.

Northern Analysis

Primary transformants containing dicots vector 2)., 3)., and 5). were analysed by northern blot analysis - using a CTP2 EPSPS probe as a *NcoI Sac* I fragment. Primary transformants containing the dicot vectors 1). 3). were analysed by northern blotting using a CTPI GOX probe as a *NcoI KpnI* fragment. Similarly, transgenic corn lines containing monocot vectors 1). and 2). were analysed using a CTP2 EPSPS probe.

Transformants containing dicot vector 5). or monocot vector 2). were treated with a foliar application of 5% ethanol to induce GOX levels. RNA was isolated 24 hours after treatment and subjected to northern analysis with a CTPI GOX probe.

Primary transformants which were PCR positive for the appropriate cassettes and showed GOX or EPSPS transcript levels were taken for further analysis.

Glyphosate Oxidoreductase Assay

Assays for glyphosate oxidoreductase were carried out as described by Kishore and Barry (WO 92/00377). These entailed measuring glyphosate - dependent uptake of oxygen

using an oxygen electrode, detection of glyoxylate formation by reaction with 2, 4 - dinitrophenylhydrazine and determination of the hydrozone using HPLC or, preferably, using [3- ^{14}C] - glyphosate as the substrate and detecting the formation of radioactive aminomethyl phosphonic acid via HPLC on an anion exchange column.

EPSPS Assay

Assays for 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase activity in plant extracts were carried out (1) by following the disappearance of the phosphoenol pyruvate substrate (as described by Rubin, J.L., Gaines, C.G and Jensen, R.A., in Plant Physiol (1984 *75*, 839-845) or (2) by conducting the assay in the reverse direction and coupling to pyruvate kinase and lactate dehydrogenase (as described by Mousdale D.M. and Coggins J.R. in Planta (1984) *160*, 78-83) or (3) by using 14(-labelled phosphoenol pyruvate as substrate and detecting the formation of radioactive EPSP by HPLC on an anion exchange column and detecting using a flow-through radioactivity detector as described by Della-Cioppa *et al* in Proc. Nat. Acad. Sci. (USA) (1986), 83, 6873-6877. The latter assay was used to confirm that the EPSP synthase activity was, as expected, relatively resistant to inhibition by glyphosate.

CLAIMS

1. A chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.

2. A chemically inducible plant gene expression cassette as claimed in claim 1, wherein the herbicide is N-phosphonomethyl-glycine or a salt or derivative thereof.

3. A chemically inducible plant gene expression cassette as claimed in claim 1 or claim 2, wherein the inducible promoter is the tetracycline repressor gene switch, the Lac repressor system, a copper inducible systems such as that based on ACE 1, a salicylic acid inducible promoters, for example the PR-1a system, a system based on a sterioid hormone such as the glucocorticoid, progesterone and oestrogen receptor systems or a modification of one of these such as a glucocorticoid receptor system which includes the GAL 4 binding domain from yeast and the VP16 activator, an insect steroid hormones systems such as that based on the ecdysone receptor of *Heliothis virescens*, a benzene sulphonamide gene switching system, a gene switching based on the alcR protein from *Aspergillus nidulans* or a glutathione S-transferase promoter.

4. A chemically inducible plant gene expression cassette comprising a first promoter operatively linked to an *alc*R regulator sequence which encodes an *alc*R regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

5. A plant gene expression cassette according to claim 4, wherein the inducible promoter is derived from the *alc*A, *alc*R, *ald*A or other *alc*R-induced gene promoter.

6. A plant gene expression cassette according to either of claims 4 and 5, wherein the inducible promoter is a chimeric promoter.

7. A plant gene expression cassette according to any preceding claim, wherein the target gene confers resistance to the herbicide N-phosphonomethyl-glycine or a salt or derivative thereof.

8. A plant cell containing a plant gene expression cassette according to any preceding claim.

9. A plant cell according to claim 8, wherein the plant gene expression cassette is stably incorporated in the plant's genome.

10. A plant tissue comprising a plant cell according to either of claims 8 and 9.

11. A plant comprising a plant cell according to either of claims 8 and 9.

12. A plant derived from a plant according to claim 11.

13. A seed derived from a plant according to either of claims 11 and 12.

14. A method of controlling herbicide resistance comprising transforming a plant cell with the plant gene expression cassette of any one of claims 1 to 7.

15. A method of selectively controlling weeds in a field of plants according to either of claims 11 or 12, or seeds according to claim 13, comprising applying an effective amount of the herbicide and the exogenous inducer.


Cat activity ng/ml/g protein
18000.00
16000.00
14000.00
12000.00
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
Time (hours)
A 0hrs
A 1hrs
A 2hrs
A 3hrs
A 4hrs
A 6hrs
A 8hrs
A 23hrs
A 48hrs
B 0hrs
B 1hrs
B 2hrs
B 3hrs
B 4hrs
B 6hrs
B 8hrs
B 23hrs
B 48hrs
C 0hrs
C 1hrs
C 2hrs
C 3hrs
C 4hrs
C 6hrs
C 8hrs
C 23hrs
C 48hrs
D 0hrs
D 1hrs
D 2hrs
D 3hrs
D 4hrs
D 6hrs
D 8hrs
D 23hrs
D 48hrs
E 0hrs
E 1hrs
E 2hrs
E 3hrs
E 4hrs
E 6hrs
E 8hrs
E 23hrs
E 48hrs
F 0hrs
F 1hrs
F 2hrs
F 3hrs
F 4hrs
F 6hrs
F 8hrs
F 23hrs
F 48hrs

Fig.1.


CAT activity ng/ml/g protein
350000.00
300000.00
250000.00
200000.00
150000.00
100000.00
50000.00
0.00
Chemical
1A
1C
2B
3A
3C
4B
5A
5C
6B
7A
7C
8B
9A
9C
10B
11A
11C
12B
13A
13C
14B
15A
15C
16B
17A
17C
20B
21A
21C
22B
23A
23C
ETOH B
acetone a
acetone c
dH20 B

Fig.2.

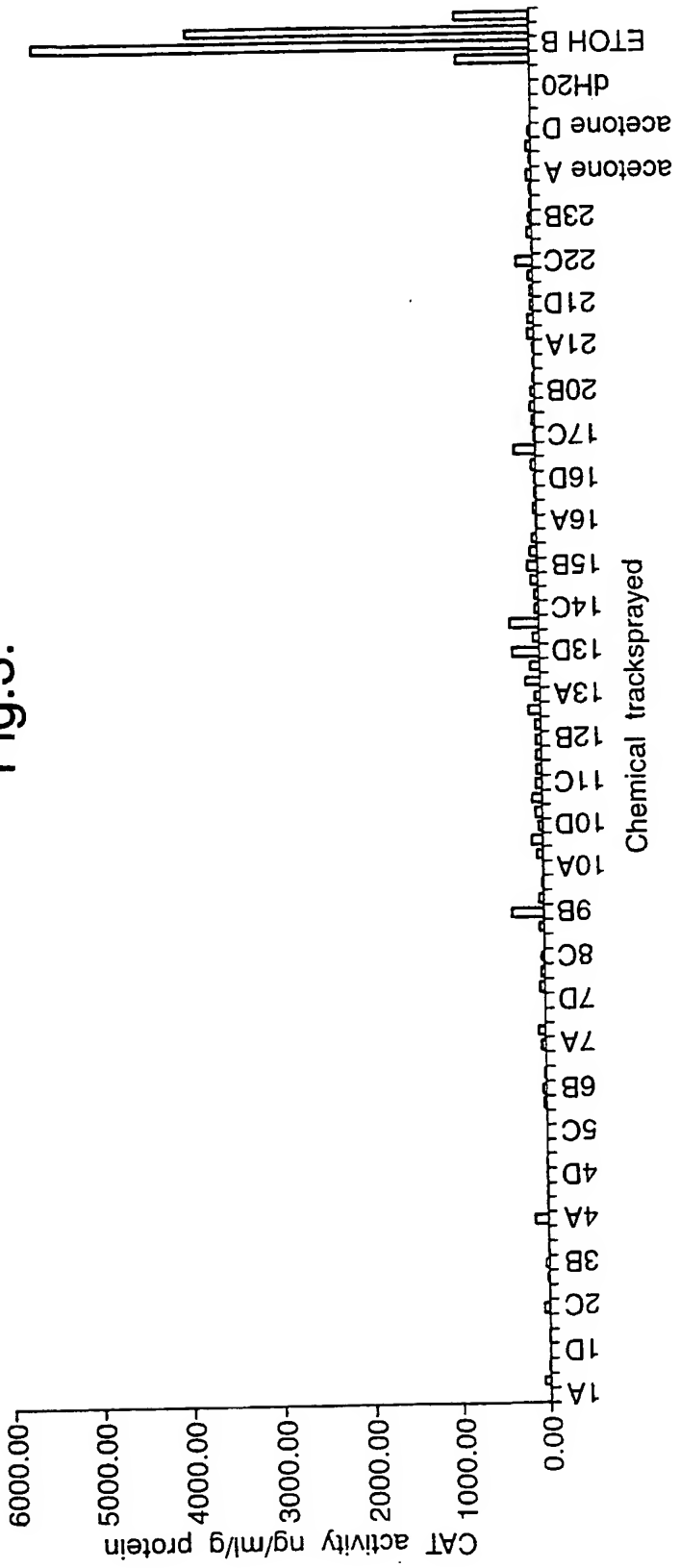

CAT activity ng/ml/g protein
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
Chemical tracksprayed
1A
1D
2C
3B
4A
4D
5C
6B
7A
7D
8C
9B
10A
10D
11C
12B
13A
13D
14C
15B
16A
16D
17C
20B
21A
21D
22C
23B
acetone A
acetone D
dH20
ETOH B

Fig.3.

Fig.4(1/2).

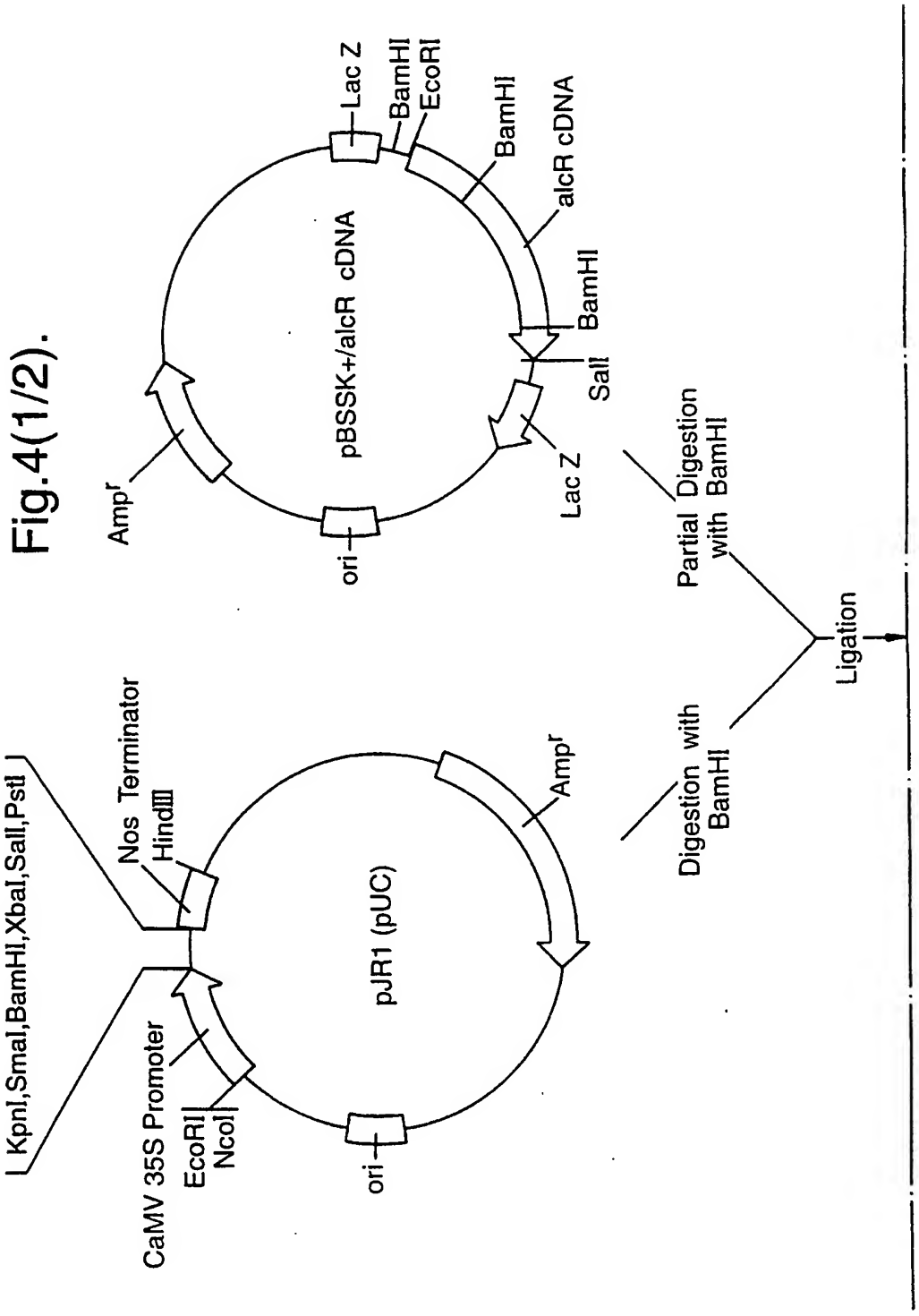

Amp^r
pBSSK+/alcR cDNA
ori
Lac Z
BamHI
EcoRI
BamHI
alcR cDNA
BamHI
SalI
Lac Z
Partial Digestion with BamHI
Ligation
Digestion with BamHI
Amp^r
pJR1 (pUC)
ori
CaMV 35S Promoter
EcoRI
NcoI
Nos Terminator
HindIII
KpnI,SmaI,BamHI,XbaI,SalI,PstI

Fig.4(2/2).

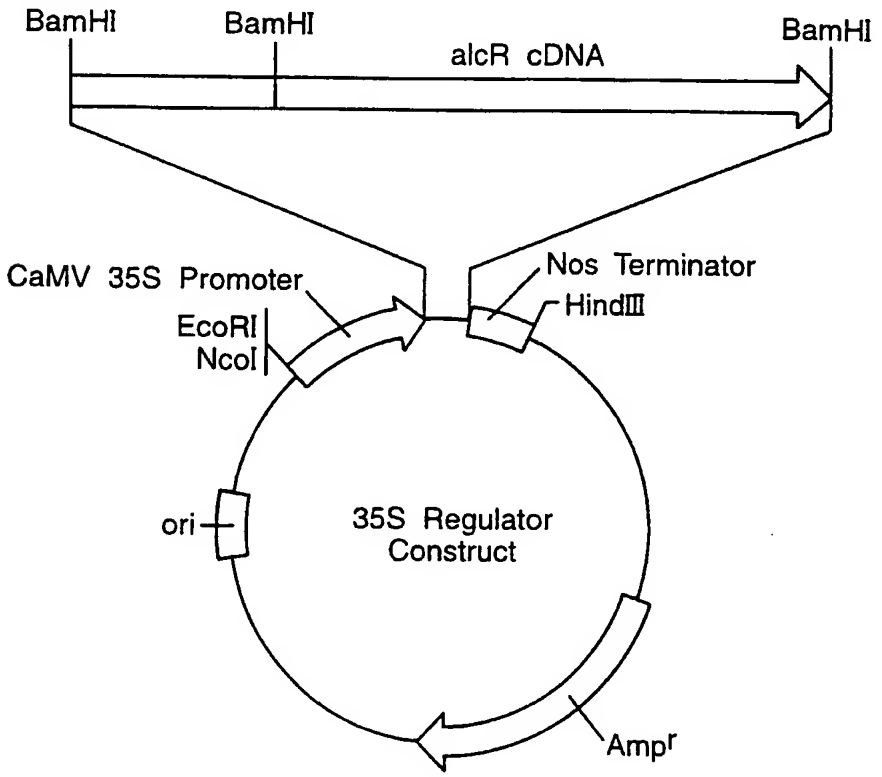
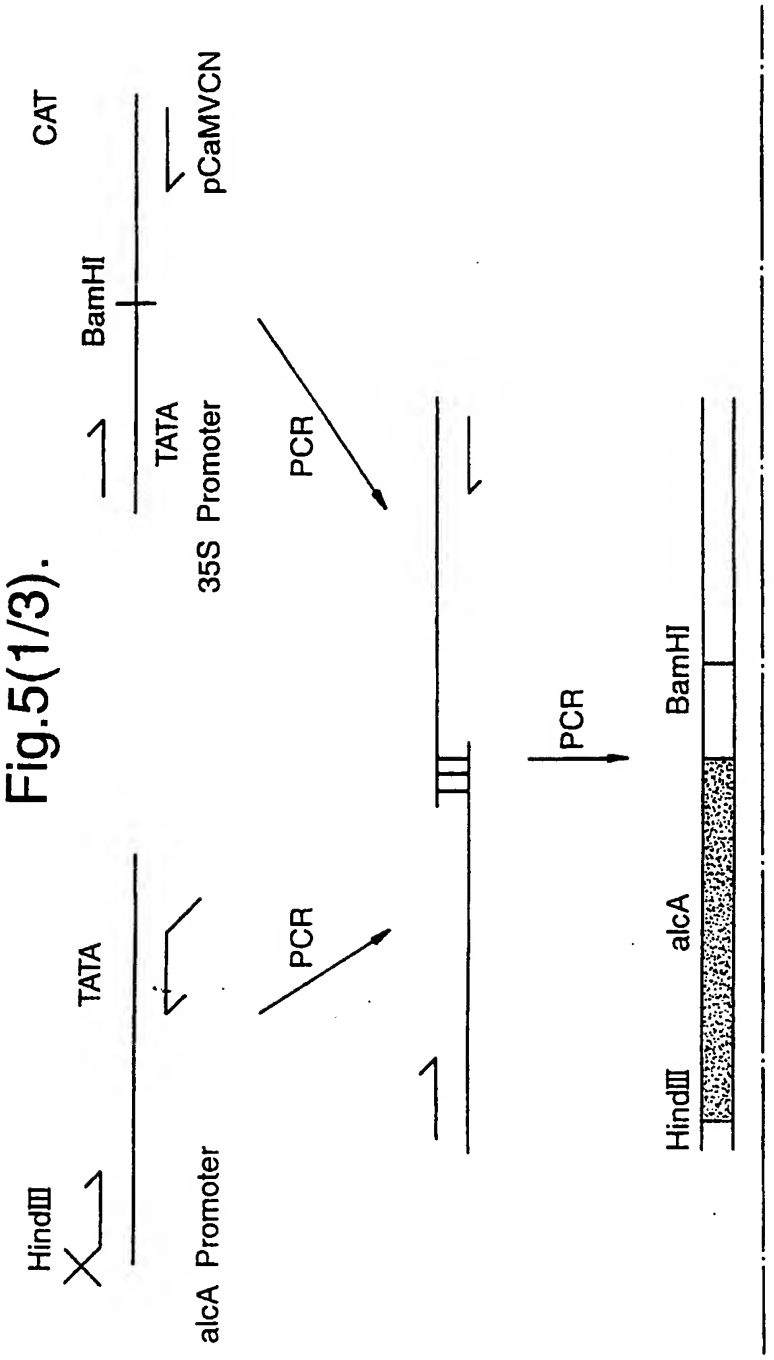

BamHI
BamHI
alcR cDNA
BamHI
CaMV 35S Promoter
EcoRI
NcoI
Nos Terminator
HindIII
ori
35S Regulator
Construct
Ampr

Fig.5(1/3).


HindIII
TATA
alcA Promoter
PCR
BamHI
TATA
35S Promoter
CAT
pCaMVCN
PCR
PCR
BamHI
alcA
HindIII

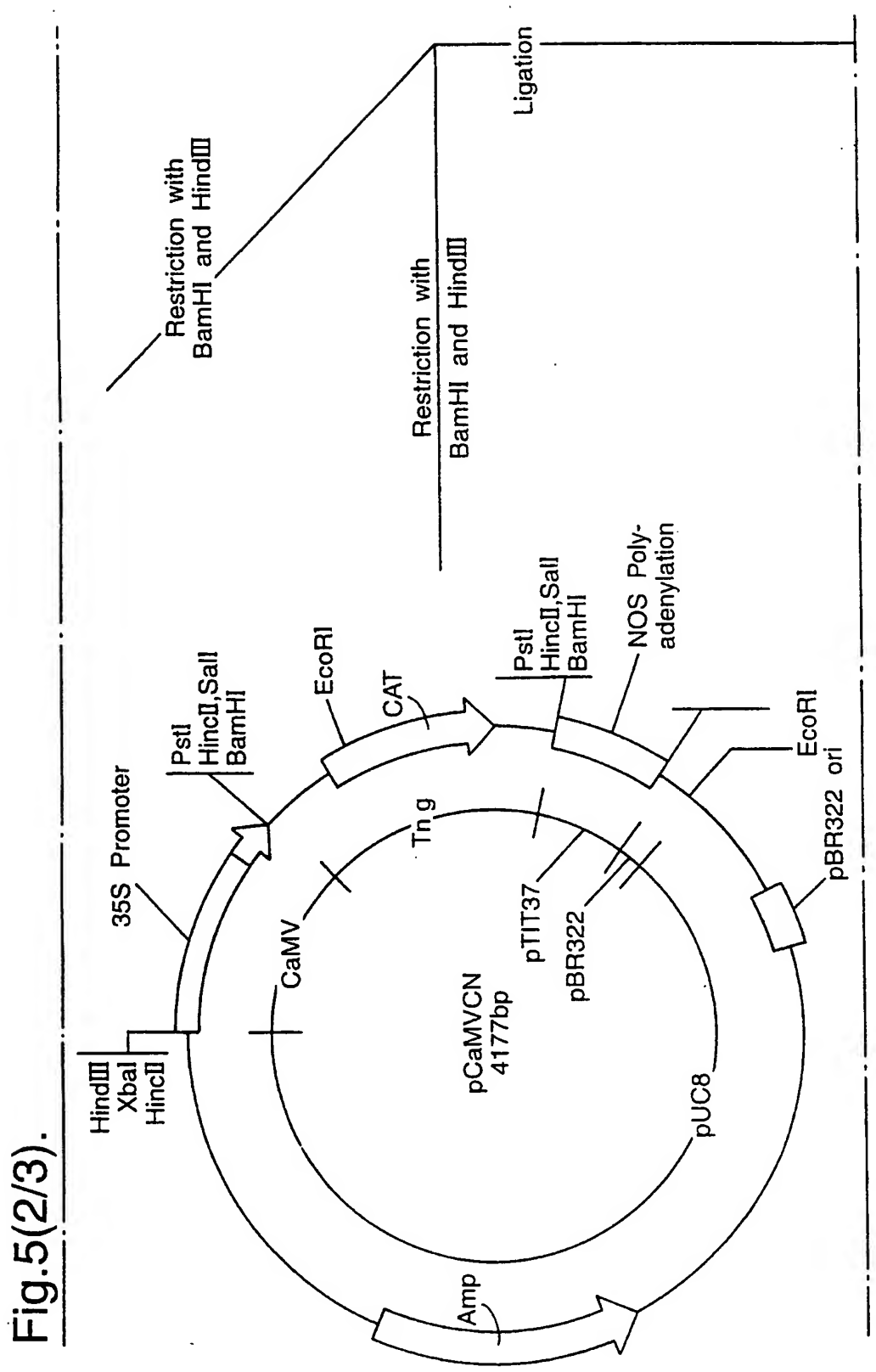

Restriction with BamHI and HindIII
Restriction with BamHI and HindIII
Ligation
35S Promoter
PstI HincII,SalI BamHI
EcoRI
CAT
PstI HincII,SalI BamHI
NOS Poly-adenylation
EcoRI
pBR322 ori
HindIII XbaI HincII
CaMV
Tn g
pTIT37
pBR322
pCaMVCN 4177bp
pUC8
Amp

Fig.5(2/3).

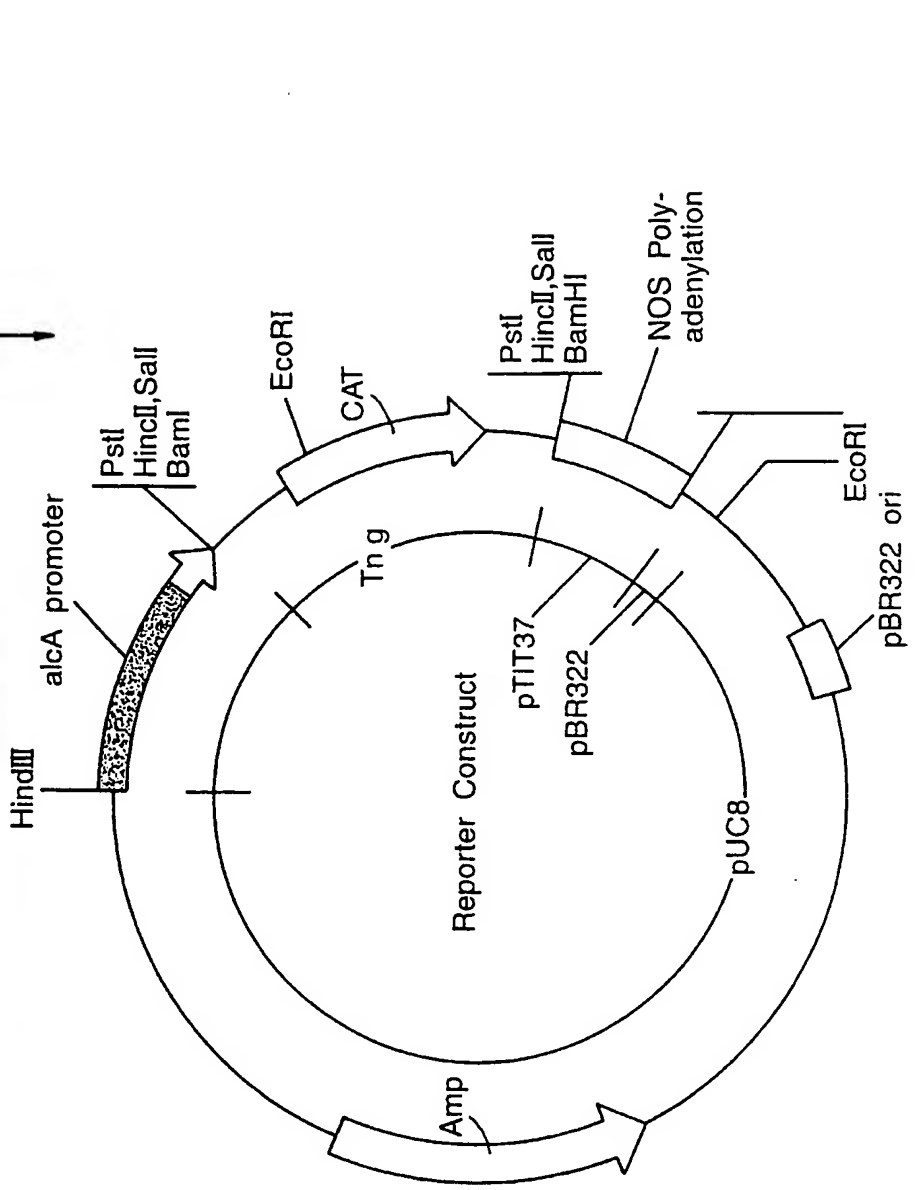
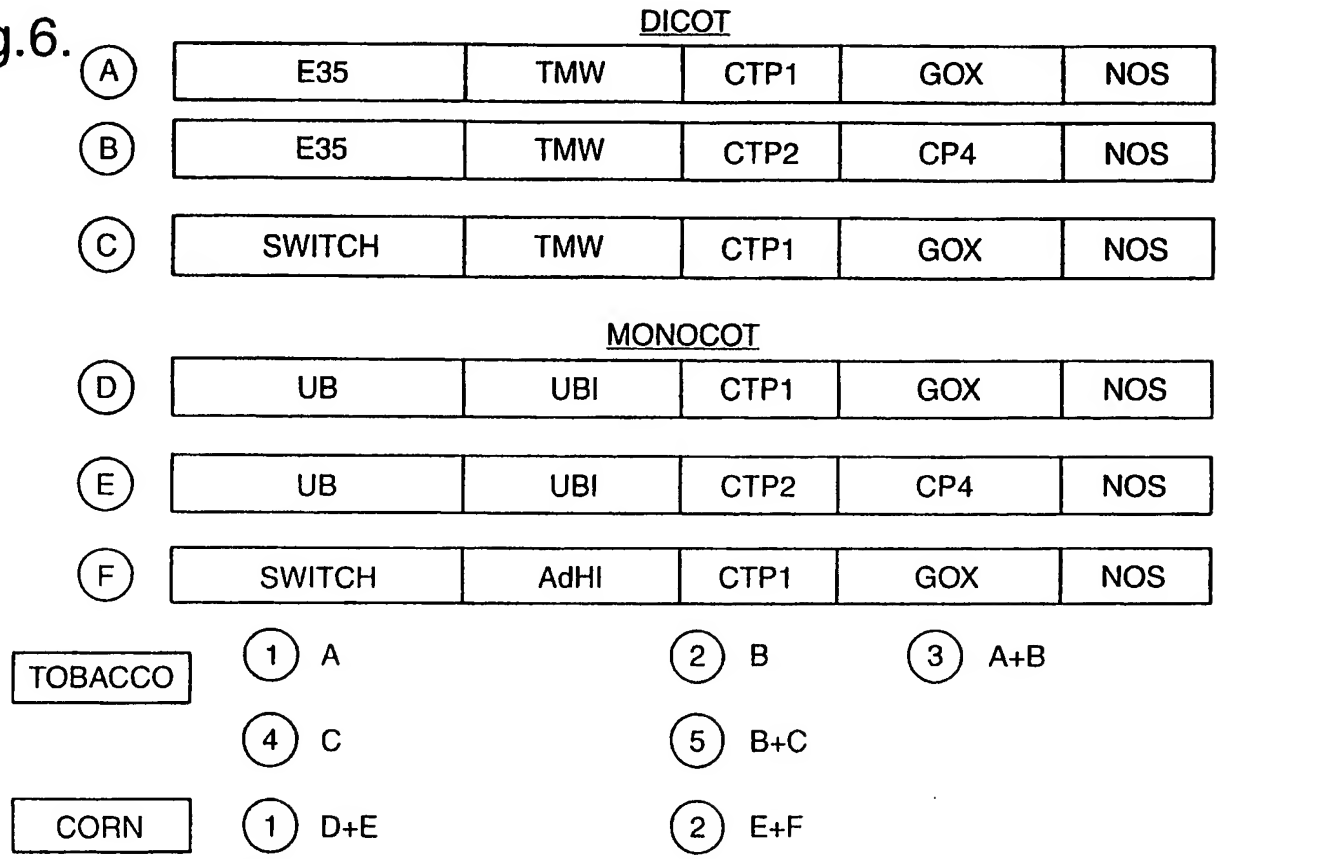

HindIII
alcA promoter
PstI
HincII,SalI
BamI
EcoRI
CAT
PstI
HincII,SalI
BamHI
NOS Poly-
adenylation
EcoRI
pBR322 ori
Tn g
pTIT37
pBR322
Reporter Construct
pUC8
Amp

Fig.5(3/3).

Fig.6.



E 35=ENHANCED CaMV35
TMV=TMV ENHANCER
CTP1=CHLOROPLAST TRANSIT SEQUENCE 1 (ARABIDOPSIS RUBISCO)
CTP2=CHLOROPLAST TRANSIT SEQUENCE EPSPS CLASS 1. PETUNIA
UB=MAIZE POLYUBIQUITIN PROMOTER
UBI=MAIZE POLYUBIQUITIN PROMOTER
AdHI=ALCOHOL DEHYDROGENASE

Fig.7(1/3).

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AAGCTTACCA TGGCTTCCTC TATGCTCTCT TCCGCTACTA TGGTTGCCTC      50
K  L  T  M  A  S  S  M  L  S  S  A  T  M  V  A  S

TCCGGCTCAG GCCACTATGG TCGCTCCTTT CAACGGACTT AAGTCCTCCG     100
 P  A  Q  A  T  M  V  A  P  F  N  G  L  K  S  S  A

CTGCCTTCCC AGCCACCCGC AAGGCTAACA ACGACATTAC TTCCATCACA     150
  A  F  P  A  T  R  K  A  N  N  D  I  T  S  I  T

AGCAACGGCG GAAGAGTTAA CTGTATGCAG GTGTGGCCTC CGATTGGAAA     200
S  N  G  G  R  V  N  C  M  Q  V  W  P  P  I  G  K

GAAGAAGTTT GAGACTCTCT CTTACCTTCC TGACCTTACC GATTCCGGTG     250
 K  K  F  E  T  L  S  Y  L  P  D  L  T  D  S  G  G

GTCGCGTCAA CTGTATGCAG GCTATGGCTG AGAACCACAA GAAGGTTGGT     300
  R  V  N  C  M  Q  A  M  A  E  N  H  K  K  V  G

ATCGCTGGAG CTGGAATCGT TGGTGTTTGC ACTGCTTTGA TGCTTCAACG     350
I  A  G  A  G  I  V  G  V  C  T  A  L  M  L  Q  R

TCGTGGATTC AAGGTTACCT TGATTGATCC AAACCCACCA GGTGAAGGTG     400
 R  G  F  K  V  T  L  I  D  P  N  P  P  G  E  G  A

CTTCTTTCGG TAACGCTGGT TGCTTCAACG GTTCCTCCGT TGTTCCAATG     450
  S  F  G  N  A  G  C  F  N  G  S  S  V  V  P  M

TCCATGCCAG GAAACTTGAC TAGCGTTCCA AAGTGGCTTC TGGATCCTGT     500
S  M  P  G  N  L  T  S  V  P  K  W  L  L  D  P  V

TGTGAATTC                                                  509
 V  N
```

Fig.7(2/3).

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AAGCTTACGG ATCCAATGGG TCCATTGTCC ATCCGTTTCA GCTACTTTCC      50
K  L  T  D  P  M  G  P  L  S  I  R  F  S  Y  F  P

AACCATCATG CCTTGGTTGA TTCGTTTCTT GCTTGCTGGA AGACCAAACA     100
 T  I  M  P  W  L  I  R  F  L  L  A  G  R  P  N  K

AGGTGAAGGA GCAAGCTAAG GCACTCCGTA ACCTCATCAA GTCCACTGTG     150
  V  K  E  Q  A  K  A  L  R  N  L  I  K  S  T  V

CCTTTGATCA AGTCCTTGGC TGAGGAGGCT GATGCTAGCC ACCTTATCCG     200
P  L  I  K  S  L  A  E  E  A  D  A  S  H  L  I  R

TCACGAAGGT CACCTTACCG TGTACCGTGG AGAAGCAGAC TTCGCCAAGG     250
 H  E  G  H  L  T  V  Y  R  G  E  A  D  F  A  K  D

ACCGTGGAGG TTGGGAACTT CGTCGTCTCA ACGGTGTTCG TACTCAAATC     300
  R  G  G  W  E  L  R  R  L  N  G  V  R  T  Q  I

CTCAGCGCTG ATGCATTGCG TGATTTCGAT CCTAACTTGT CTCACGCCTT     350
L  S  A  D  A  L  R  D  F  D  P  N  L  S  H  A  F

TACCAAGGGA ATCCTTATCG AAGAGAACGG TCACACCATC AACCCACAAG     400
 T  K  G  I  L  I  E  E  N  G  H  T  I  N  P  Q  G

GTCTCGTGAC TCTCTTGTTT CGTCGTTTCA TCGCTAACGG TGGAGAGTTC     450
  L  V  T  L  L  F  R  R  F  I  A  N  G  G  E  F

GTGTCTGCTC GTGTTATCGG ATTCGAGACT GAAGGTCGTG CTCTCAAGGG     500
V  S  A  R  V  I  G  F  E  T  E  G  R  A  L  K  G

TATCACCACC ACCAACGGTG TTCTTGCTGT TGATGCTGCA GTGTTGTGAA     550
 I  T  T  T  N  G  V  L  A  V  D  A  A  V  L  .  I

TTC                                                        553
```

Fig.7(3/3).

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AAGCTTACTG CAGTTGTTGC AGCTGGTGCA CACTCCAAGT CTCTTGCTAA      50
K  L  T  A  V  V  A  A  G  A  H  S  K  S  L  A  N

CTCCCTTGGT GATGACATCC CATTGGATAC CGAACGTGGA TACCACATCG     100
 S  L  G  D  D  I  P  L  D  T  E  R  G  Y  H  I  V

TGATCGCCAA CCCAGAAGCT GCTCCACGTA TTCCAACTAC CGATGCTTCT     150
  I  A  N  P  E  A  A  P  R  I  P  T  T  D  A  S

GGAAAGTTCA TCGCTACTCC TATGGAGATG GGTCTTCGTG TTGCTGGAAC     200
G  K  F  I  A  T  P  M  E  M  G  L  R  V  A  G  T

CGTTGAGTTC GCTGGTCTCA CTGCTGCTCC TAACTGGAAG CGTGCTCACG     250
 V  E  F  A  G  L  T  A  A  P  N  W  K  R  A  H  V

TTCTCTACAC TCGTGCTCGT AAGTTGCTTC CAGCTCTCGC TCCTGCCAGT     300
  L  Y  T  R  A  R  K  L  L  P  A  L  A  P  A  S

TCTGAAGAAC GTTACTCCAA GTGGATGGGT TTCCGTCCAA GCATCCCAGA     350
S  E  E  R  Y  S  K  W  M  G  F  R  P  S  I  P  D

TTCCCTTCCA GTGATTGGTC GTGCTACCCG TACTCCAGAC GTTATCTACG     400
 S  L  P  V  I  G  R  A  T  R  T  P  D  V  I  Y  A

CTTTCGGTCA CGGTCACCTC GGTATGACTG GTGCTCCAAT GACCGCAACC     450
  F  G  H  G  H  L  G  M  T  G  A  P  M  T  A  T

CTCGTTTCTG AGCTCCTCGC AGGTGAGAAG ACCTCTATCG ACATCTCTCC     500
L  V  S  E  L  L  A  G  E  K  T  S  I  D  I  S  P

ATTCGCACCA AACCGTTTCG GTATTGGTAA GTCCAAGCAA ACTGGTCCTG     550
 F  A  P  N  R  F  G  I  G  K  S  K  Q  T  G  P  A

CATCCTAAGG TACCGAATTC                                     570
  S  .  G  T  E  F
```

Fig.8.

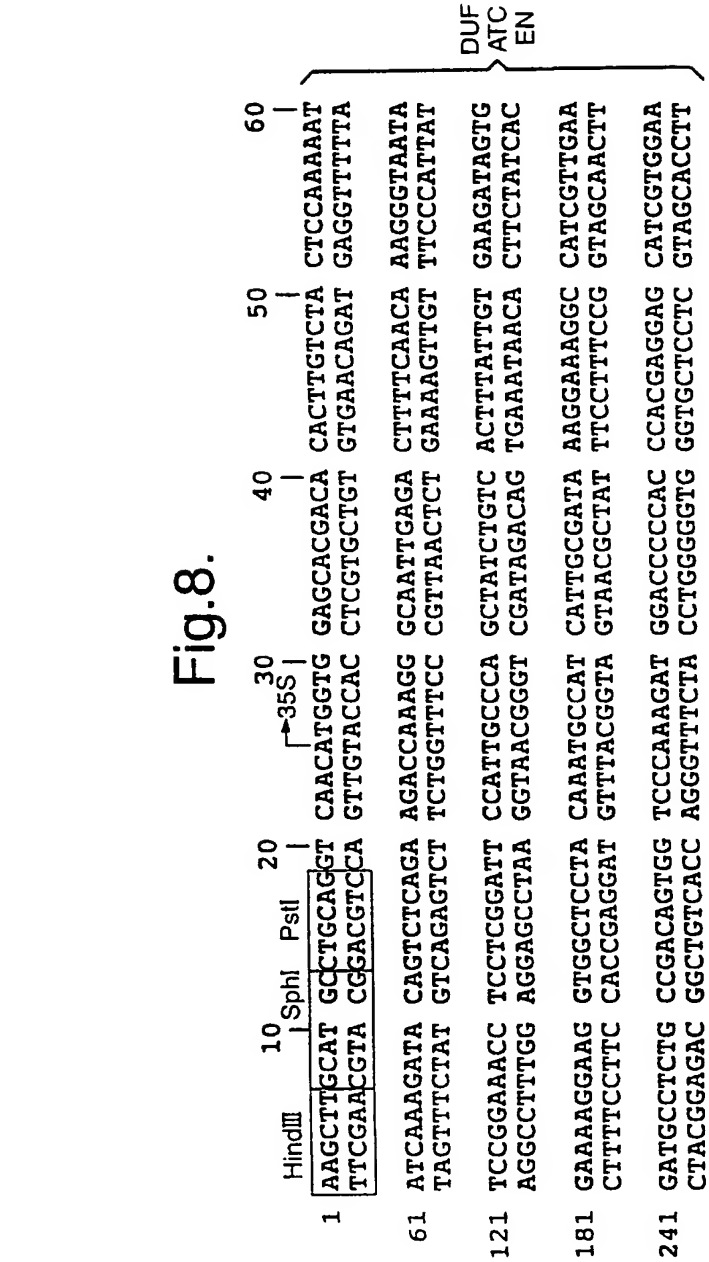

HindIII
SphI
PstI
35S
DUF ATC EN
1 AAGCTTGCATGCCTGCAGGT CAACATGGTG GAGCACGACA CACTTGTCTA CTCCAAAAAT
TTCGAACGTACGGACGTCCA GTTGTACCAC CTCGTGCTGT GTGAACAGAT GAGGTTTTTA
61 ATCAAAGATA CAGTCTCAGA AGACCAAAGG GCAATTGAGA CTTTTCAACA AAGGGTAATA
TAGTTTCTAT GTCAGAGTCT TCTGGTTTCC CGTTAACTCT GAAAAGTTGT TTCCCATTAT
121 TCCGGAAACC TCCTCGGATT CCATTGCCCA GCTATCTGTC ACTTTATTGT GAAGATAGTG
AGGCCTTTGG AGGAGCCTAA GGTAACGGGT CGATAGACAG TGAAATAACA CTTCTATCAC
181 GAAAAGGAAG GTGGCTCCTA CAAATGCCAT CATTGCGATA AAGGAAAGGC CATCGTTGAA
CTTTTCCTTC CACCGAGGAT GTTTACGGTA GTAACGCTAT TTCCTTTCCG GTAGCAACTT
241 GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGAGGAG CATCGTGGAA
CTACGGAGAC GGCTGTCACC AGGGTTTCTA CCTGGGGGTG GGTGCTCCTC GTAGCACCTT

Fig.8 (Cont).

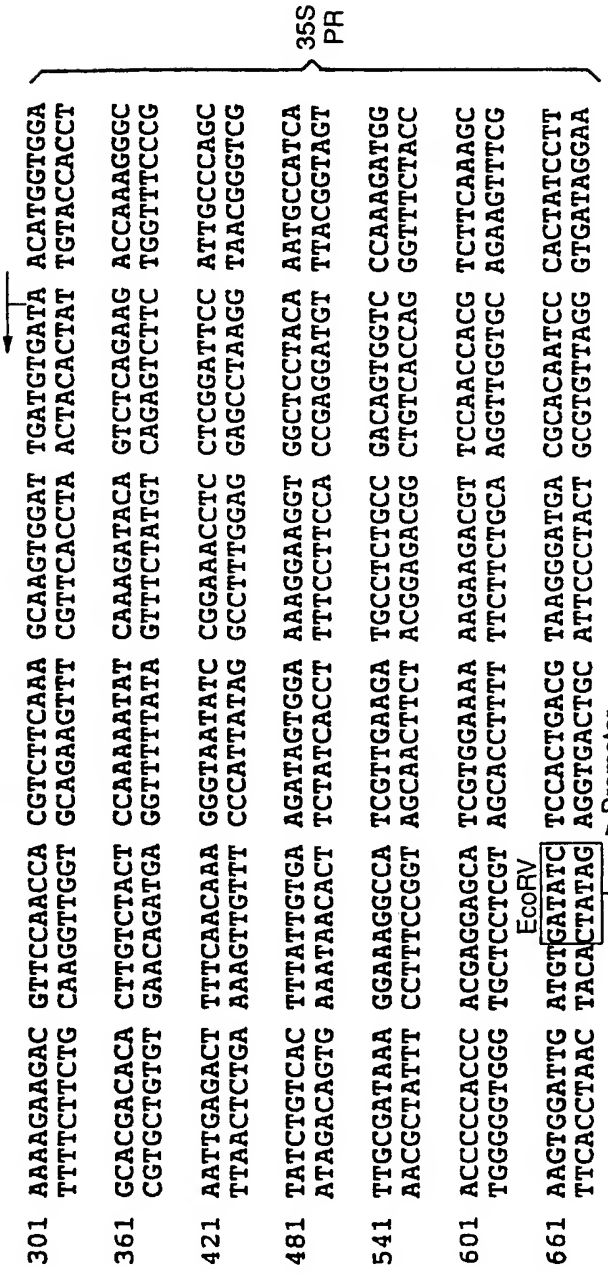

35S PR
301 AAAAGAAGAC GTTCCAACCA CGTCTTCAAA GCAAGTGGAT TGATGTGATA ACATGGTGGA
TTTTCTTCTG CAAGGTTGGT GCAGAAGTTT CGTTCACCTA ACTACACTAT TGTACCACCT
361 GCACGACACA CTTGTCTACT CCAAAAATAT CAAAGATACA GTCTCAGAAG ACCAAAGGGC
CGTGCTGTGT GAACAGATGA GGTTTTTATA GTTTCTATGT CAGAGTCTTC TGGTTTCCCG
421 AATTGAGACT TTTCAACAAA GGGTAATATC CGGAAACCTC CTCGGATTCC ATTGCCCAGC
TTAACTCTGA AAAGTTGTTT CCCATTATAG GCCTTTGGAG GAGCCTAAGG TAACGGGTCG
481 TATCTGTCAC TTTATTGTGA AGATAGTGGA AAAGGAAGGT GGCTCCTACA AATGCCATCA
ATAGACAGTG AAATAACACT TCTATCACCT TTTCCTTCCA CCGAGGATGT TTACGGTAGT
541 TTGCGATAAA GGAAAGGCCA TCGTTGAAGA TGCCTCTGCC GACAGTGGTC CCAAAGATGG
AACGCTATTT CCTTTCCGGT AGCAACTTCT ACGGAGACGG CTGTCACCAG GGTTTCTACC
601 ACCCCCACCC ACGAGGAGCA TCGTGGAAAA AAGAAGACGT TCCAACCACG TCTTCAAAGC
TGGGGGTGGG TGCTCCTCGT AGCACCTTTT TTCTTCTGCA AGGTTGGTGC AGAAGTTTCG
EcoRV
661 AAGTGGATTG ATGTGATATC TCCACTGACG TAAGGGATGA CGCACAATCC CACTATCCTT
TTCACCTAAC TACACTATAG AGGTGACTGC ATTCCCTACT GCGTGTTAGG GTGATAGGAA
Promoter

Fig. 8 (Cont i).

```
                                                                         TSP  XhoI
 721  CGCAAGACCC TTCCTCTATA TAAGGAAGTT CATTTCATTT GGAGAGGACC TCGAGTATTT
      GCGTTCTGGG AAGGAGATAT ATTCCTTCAA GTAAAGTAAA CCTCTCCTGG AGCTCATAAA
                       TATA BOX
 781  TTACAACAAT TACCAACAAC AACAAACAAC AAACAACATT ACAATTACTA TTTACAATTA   TM Ω
      AATGTTGTTA ATGGTTGTTG TTGTTTGTTG TTTGTTGTAA TGTTAATGAT AAATGTTAAT
        NcoI        SmaI     KpnI  SacI
 841  CACCATGGAT CCCCGGGTAC CGAGCTCGAA TTTCCCCGAT CGTTCAAACA TTTGGCAATA
      GTGGTACCTA GGGGCCCATG GCTCGAGCTT AAAGGGGCTA GCAAGTTTGT AAACCGTTAT
             BamHI
 901  AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGCGATG ATTATCATAT AATTTCTGTT
      TTCAAAGAAT TCTAACTTAG GACAACGGCC AGAACGCTAC TAATAGTATA TTAAAGACAA

 961  GAATTACGTT AAGCATGTAA TAATTAACAT GTAATGCATG ACGTTATTTA TGAGATGGGT   NOS 3'
      CTTAATGCAA TTCGTACATT ATTAATTGTA CATTACGTAC TGCAATAAAT ACTCTACCCA

1021  TTTTATGATT AGAGTCCCGC AATTATACAT TTAATACGCG ATAGAAAACA AAATATAGCG
      AAAATACTAA TCTCAGGGCG TTAATATGTA AATTATGCGC TATCTTTTGT TTTATATCGC

1081  CGCAAACTAG GATAAATTAT CGCGCGCGGT GTCATCTATG TTACTAGATC GGGAATTC
      GCGTTTGATC CTATTTAATA GCGCGCGCCA CAGTAGATAC AATGATCTAG CCCTTAAG
                                                               EcoRI
```

Total number of bases is: 1138.
DNA sequence composition: 370 A; 253 C; 234 G; 281 T; 0 OTHER;

Sequence name: PMJB1

Fig.9.

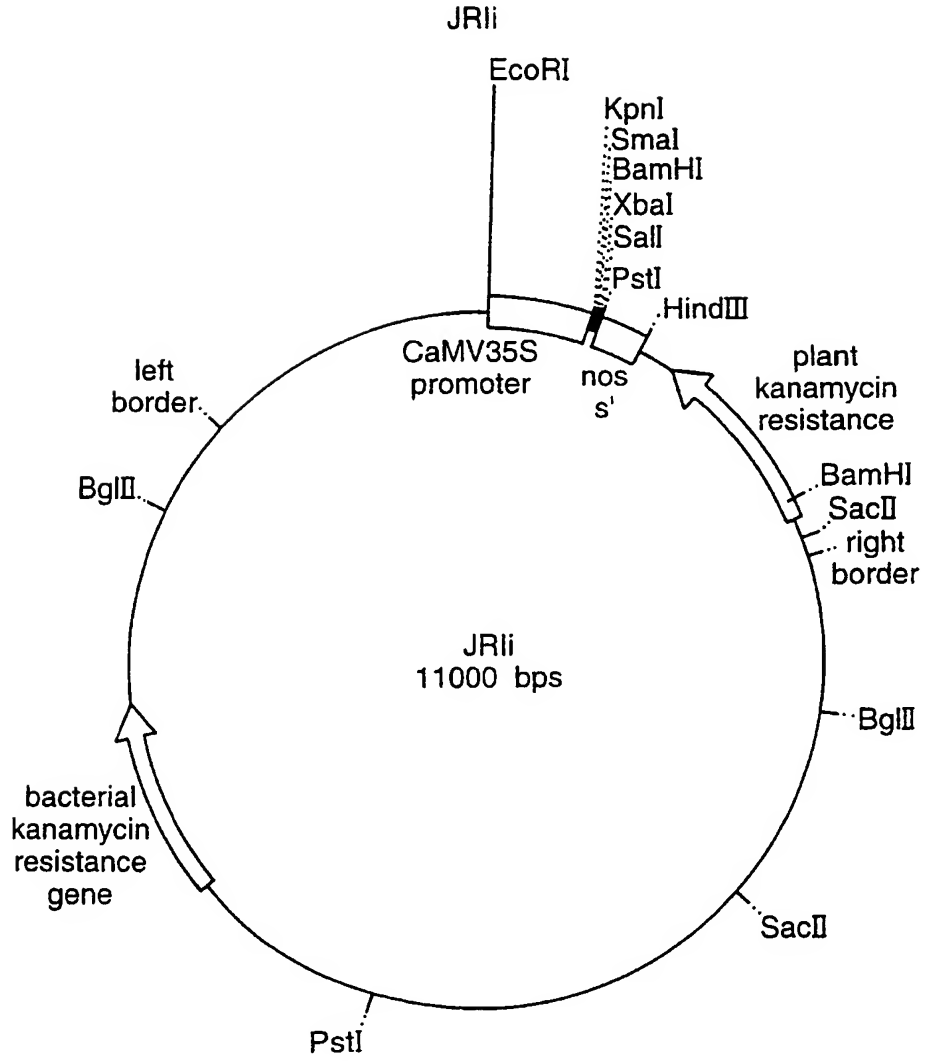

JRIi
EcoRI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
HindIII
CaMV35S promoter
nos s'
plant kanamycin resistance
BamHI
SacII
right border
BglII
SacII
PstI
bacterial kanamycin resistance gene
BglII
left border
JRIi
11000 bps

Fig.10(1/4).

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AAGCTTACCA TGGCTCAAGT TAGCAGAATC TGCAATGGTG TGCAGAACCC      50
K  L  T  M  A  Q  V  S  R  I  C  N  G  V  Q  N  P

ATCTCTTATC TCCAATCTCT CGAAATCCAG TCAACGCAAA TCTCCCTTAT     100
 S  L  I  S  N  L  S  K  S  S  Q  R  K  S  P  L  S

CGGTTTCTCT GAAGACGCAG CAGCATCCAC GAGCTTATCC GATTTCGTCG     150
  V  S  L  K  T  Q  Q  H  P  R  A  Y  P  I  S  S

TCGTGGGGAT TGAAGAAGAG TGGGATGACG TTAATTGGCT CTGAGCTTCG     200
S  W  G  L  K  K  S  G  M  T  L  I  G  S  E  L  R

TCCTCTTAAG GTCATGTCTT CTGTTTCCAC GGCGTGTATG CTTCACGGTG     250
 P  L  K  V  M  S  S  V  S  T  A  C  M  L  H  G  A

CAAGCAGCCG TCCAGCAACT GCTCGTAAGT CCTCTGGTCT TTCTGGAACC     300
  S  S  R  P  A  T  A  R  K  S  S  G  L  S  G  T

GTCCGTATTC CAGGTGACAA GTCTATCTCC CACAGGTCCT TCATGTTTGG     350
V  R  I  P  G  D  K  S  I  S  H  R  S  F  M  F  G

AGGTCTCGCT AGCGGTGAAA CTCGTATCAC CGGTCTTTTG GAAGGTGAAG     400
 G  L  A  S  G  E  T  R  I  T  G  L  L  E  G  E  D

ATGTTATCAA CACTGGTAAG GCTATGCAAG CTATGGGTGC CAGGATCCTG     450
  V  I  N  T  G  K  A  M  Q  A  M  G  A  R  I  L

TTGTGAATTC                                                 460
L  .  I
```

Fig.10(2/4). 18/30

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AAGCTTAGGA TCCGTAAGGA AGGTGATACT TGGATCATTG ATGGTGTTGG   50
K  L  R  I   R  K  E   G  D  T   W  I  I  D   G  V  G

TAACGGTGGA CTCCTTGCTC CTGAGGCTCC TCTCGATTTC GGTAACGCTG  100
N  G  G   L  L  A  P   E  A  P   L  D  F   G  N  A  A

CAACTGGTTG CCGTTTGACT ATGGGTCTTG TTGGTGTTTA CGATTTCGAT  150
  T  G  C   R  L  T   M  G  L  V   G  V  Y   D  F  D

AGCACTTTCA TTGGTGACGC TTCTCTCACT AAGCGTCCAA TGGGTCGTGT  200
S  T  F  I   G  D  A   S  L  T   K  R  P  M   G  R  V

GTTGAACCCA CTTCGCGAAA TGGGTGTGCA GGTGAAGTCT GAAGACGGTG  250
L  N  P   L  R  E  M   G  V  Q   V  K  S   E  D  G  D

ATCGTCTTCC AGTTACCTTG CGTGGACCAA AGACTCCAAC GCCAATCACC  300
  R  L  P   V  T  L   R  G  P  K   T  P  T   P  I  T

TACAGGGTAC CTATGGCTTC CGCTCAAGTG AAGTCCGCTG TTCTGCTTGC  350
Y  R  V  P   M  A  S   A  Q  V   K  S  A  V   L  L  A

TGGTCTCAAC ACCCCAGGTA TCACCACTGT TATCGAGCCA ATCATGACTC  400
G  L  N   T  P  G  I   T  T  V   I  E  P   I  M  T  R

GTGACCACAC TGAAAAGATG CTTCAAGGTT TTGGTGCTAA CCTTACCGTT  450
  D  H  T   E  K  M   L  Q  G  F   G  A  N   L  T  V

GAGACTGATG CTGACGGTGT GCGTACCATC CGTCTTGAAG GTCGTGGTAA  500
E  T  D  A   D  G  V   R  T  I   R  L  E  G   R  G  K

GCTCACCGGT CAAGTGATTG ATGTTCCAGG TGATCCATCC TCTACTGCTT  550
L  T  G   Q  V  I  D   V  P  G   D  P  S   S  T  A  F

TCCCATTGGT TGCTGCCTTG CTTGTTCCAG GTTCCGACGT CACCATCCTT  600
  P  L  V   A  A  L   L  V  P  G   S  D  V   T  I  L

AACGTTTTGA TGAACCCAAC CCGTACTGGT CTCATCTTGA CTCTGCAGTG  650
N  V  L  M   N  P  T   R  T  G   L  I  L  T   L  Q  C
```

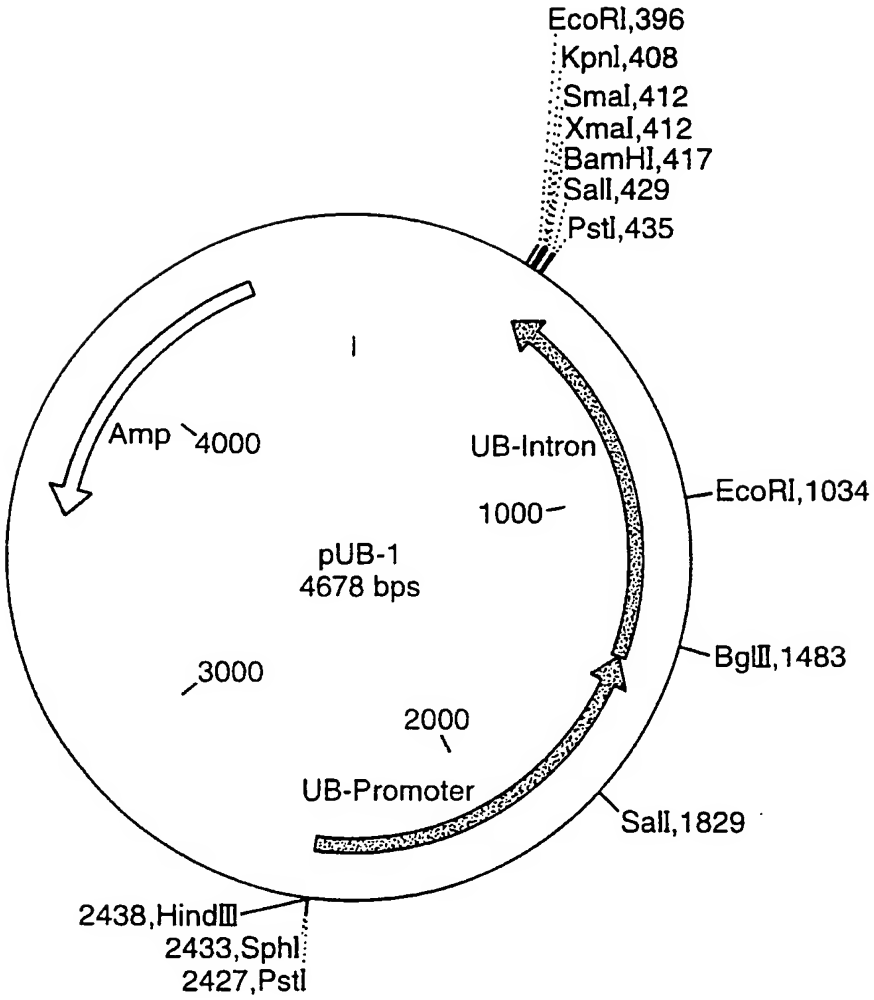
Fig.10(3/4).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TTGTGAATTC					660
C E F					

Fig.10(4/4).

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
AAGCTTCTGC AGGAAATGGG TGCCGACATC GAAGTGATCA ACCCACGTCT      50
K  L  L  Q  E  M  G  A  D  I  E  V  I  N  P  R  L

TGCTGGTGGA GAAGACGTGG CTGACTTGCG TGTTCGTTCT TCTACTTTGA     100
 A  G  G  E  D  V  A  D  L  R  V  R  S  S  T  L  K

AGGGTGTTAC TGTTCCAGAA GACCGTGCTC CTTCTATGAT CGACGAGTAT     150
  G  V  T  V  P  E  D  R  A  P  S  M  I  D  E  Y

CCAATTCTCG CTGTTGCAGC TGCATTCGCT GAAGGTGCTA CCGTTATGAA     200
P  I  L  A  V  A  A  A  F  A  E  G  A  T  V  M  N

CGGTTTGGAA GAACTCCGTG TTAAGGAAAG CGACCGTCTT TCTGCTGTCG     250
 G  L  E  E  L  R  V  K  E  S  D  R  L  S  A  V  A

CAAACGGTCT CAAGCTCAAC GGTGTTGATT GCGATGAAGG TGAGACTTCT     300
  N  G  L  K  L  N  G  V  D  C  D  E  G  E  T  S

CTCGTCGTGC GTGGTCGTCC TGACGGTAAG GGTCTCGGTA ACGCTTCTGG     350
L  V  V  R  G  R  P  D  G  K  G  L  G  N  A  S  G

AGCAGCTGTC GCTACCCACC TCGATCACCG TATCGCTATG AGCTTCCTCG     400
 A  A  V  A  T  H  L  D  H  R  I  A  M  S  F  L  V

TTATGGGTCT CGTTTCTGAA AACCCTGTTA CTGTTGATGA TGCTACTATG     450
  M  G  L  V  S  E  N  P  V  T  V  D  D  A  T  M

ATCGCTACTA GCTTCCCAGA GTTCATGGAT TTGATGGCTG GTCTTGGAGC     500
I  A  T  S  F  P  E  F  M  D  L  M  A  G  L  G  A

TAAGATCGAA CTCTCCGACA CTAAGGCTGC TTGATGAGCT CGAATTC        547
 K  I  E  L  S  D  T  K  A  A  .  .  A  R  I
```

Fig.11.



Ubiquitin promoter fragment PCRed from maize.
2 Kb. fragment cloned into pUC19.
Junctions have been sequenced to confirm that it is the Ubiquitin promoter.

Fig.12.

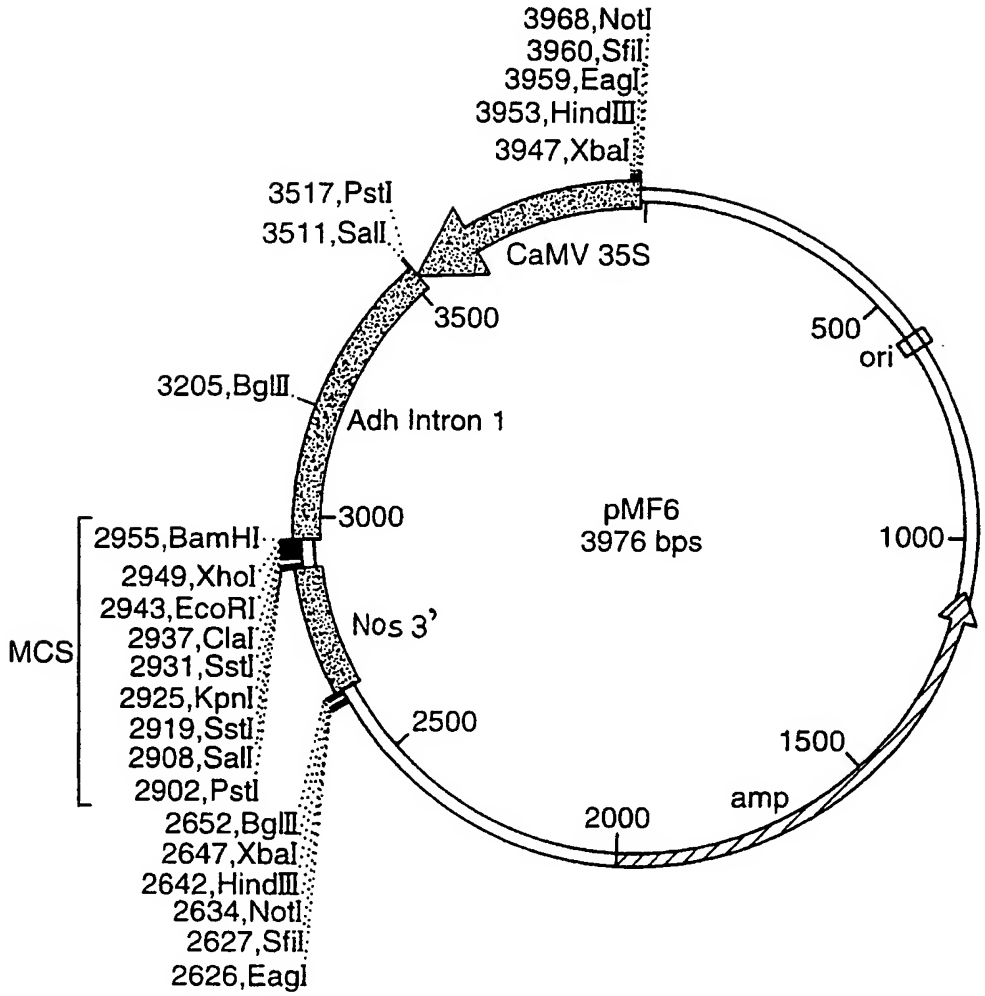

3968,NotI
3960,SfiI
3959,EagI
3953,HindIII
3947,XbaI
3517,PstI
3511,SalI
CaMV 35S
3500
500
ori
3205,BglII
Adh Intron 1
3000
pMF6
3976 bps
1000
2955,BamHI
2949,XhoI
2943,EcoRI
2937,ClaI
2931,SstI
2925,KpnI
2919,SstI
2908,SalI
2902,PstI
MCS
Nos 3'
2500
1500
amp
2000
2652,BglII
2647,XbaI
2642,HindIII
2634,NotI
2627,SfiI
2626,EagI

Fig.13.

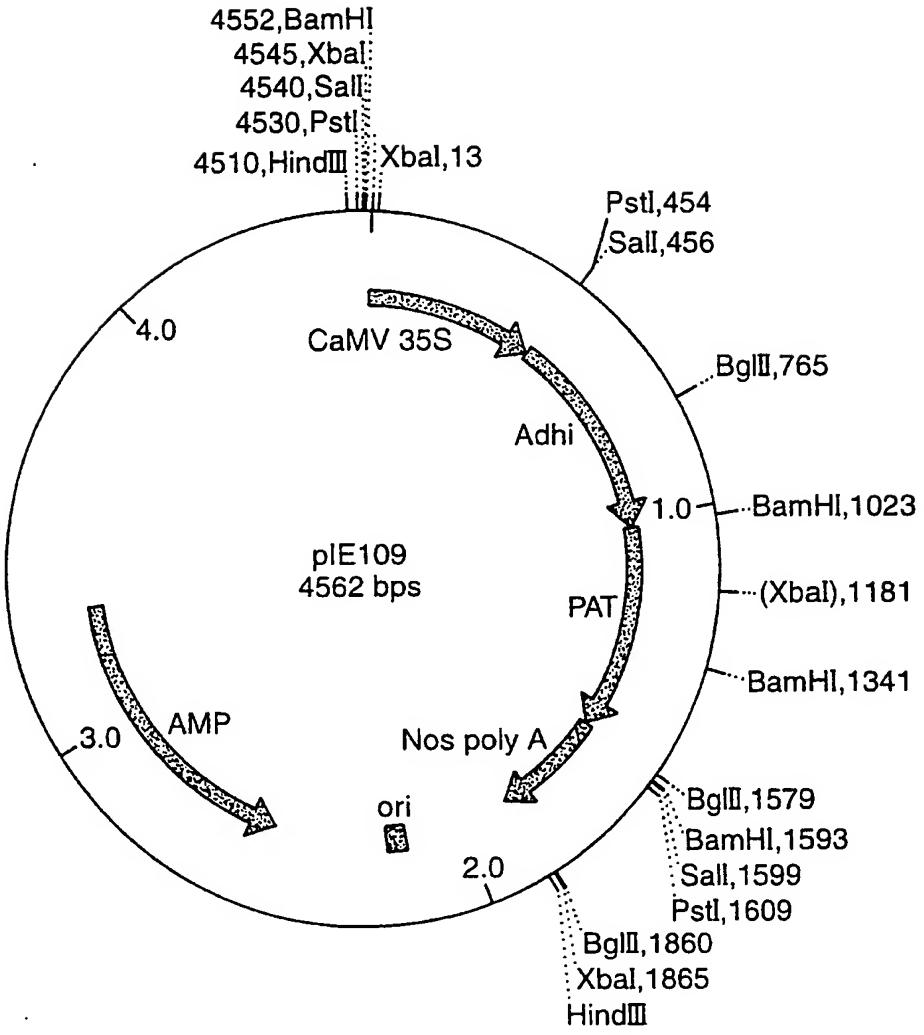

4552,BamHI
4545,XbaI
4540,SalI
4530,PstI
4510,HindIII
XbaI,13
PstI,454
SalI,456
4.0
CaMV 35S
BglII,765
Adhi
1.0
BamHI,1023
pIE109
4562 bps
(XbaI),1181
PAT
BamHI,1341
AMP
3.0
Nos poly A
ori
BglII,1579
BamHI,1593
SalI,1599
PstI,1609
2.0
BglII,1860
XbaI,1865
HindIII

Fig.14.

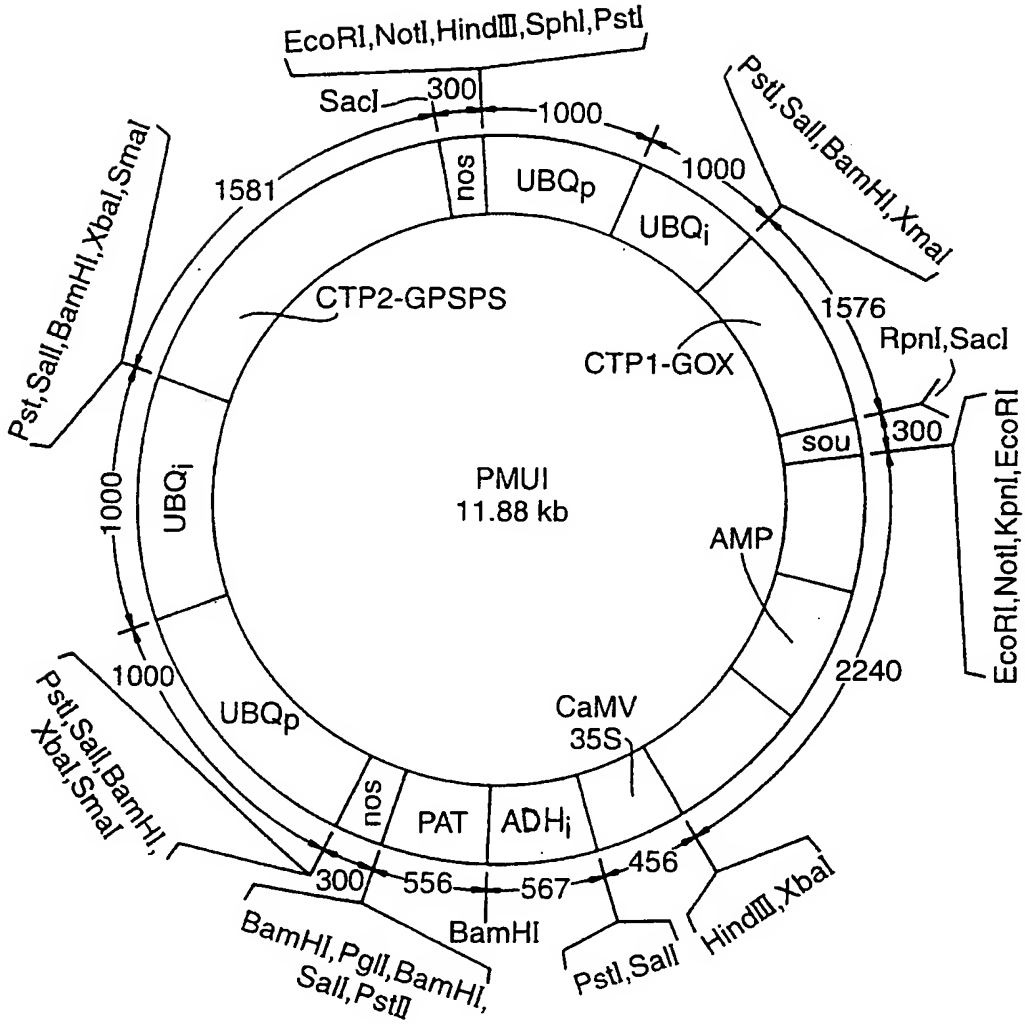

EcoRI,NotI,HindIII,SphI,PstI
SacI
300
1000
1000
PstI,SalI,BamHI,XmaI
1581
nos
UBQp
UBQi
CTP2-GPSPS
1576
CTP1-GOX
RpnI,SacI
sou
300
Pst,SalI,BamHI,XbaI,SmaI
1000
UBQi
PMUI
11.88 kb
AMP
EcoRI,NotI,KpnI,EcoRI
2240
1000
UBQp
CaMV
35S
PstI,SalI,BamHI,
XbaI,SmaI
nos
PAT
ADHi
300
556
567
456
BamHI
BamHI,PgII,BamHI,
SalI,PstII
PstI,SalI
HindIII,XbaI

Fig.15.

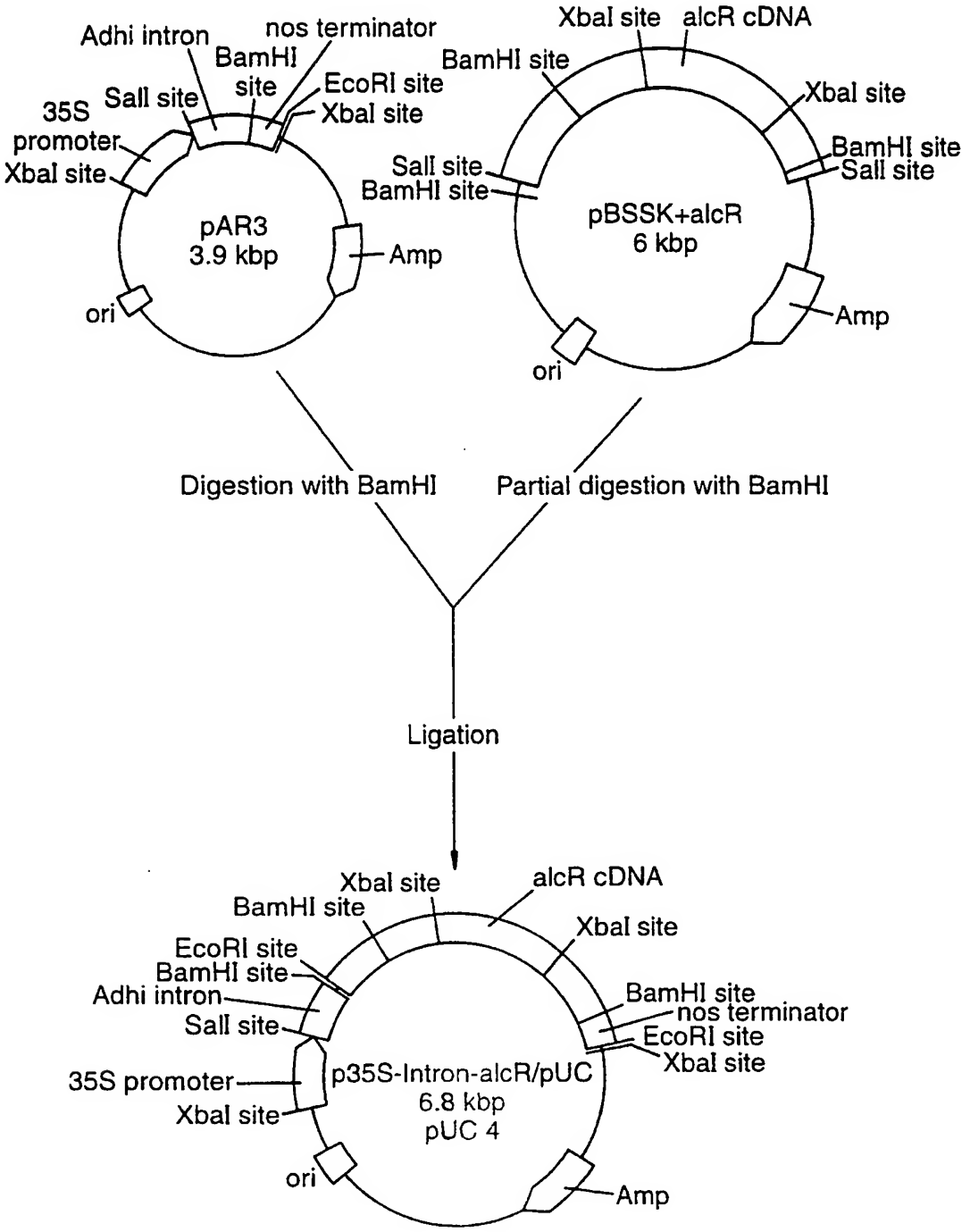

Adhi intron
nos terminator
BamHI site
EcoRI site
SalI site
XbaI site
35S promoter
XbaI site
pAR3
3.9 kbp
Amp
ori
XbaI site
alcR cDNA
BamHI site
XbaI site
BamHI site
SalI site
SalI site
BamHI site
pBSSK+alcR
6 kbp
Amp
ori
Digestion with BamHI
Partial digestion with BamHI
Ligation
XbaI site
alcR cDNA
BamHI site
XbaI site
EcoRI site
BamHI site
BamHI site
Adhi intron
nos terminator
SalI site
EcoRI site
XbaI site
35S promoter
p35S-Intron-alcR/pUC
6.8 kbp
pUC 4
XbaI site
ori
Amp

Fig.16.

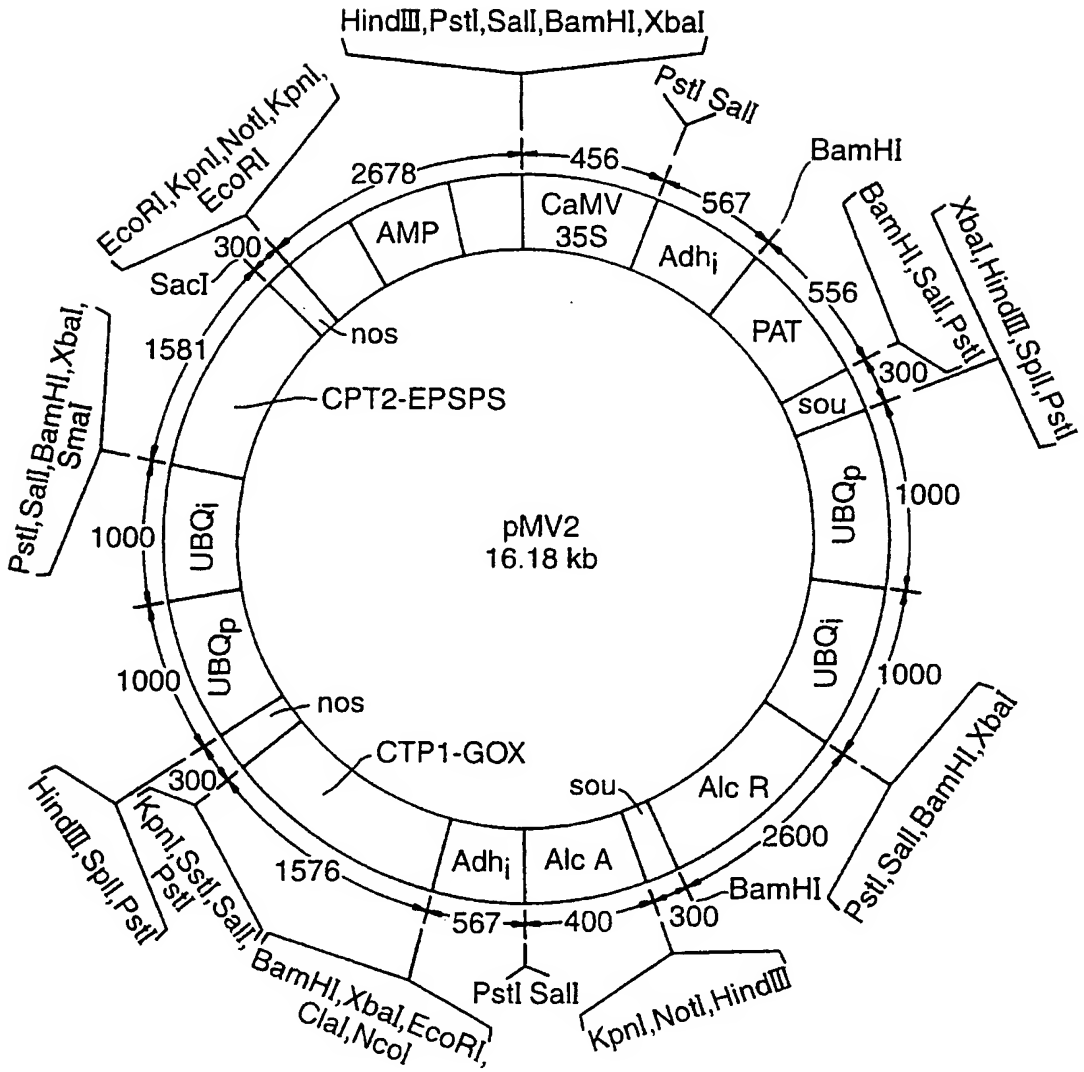

HindIII,PstI,SalI,BamHI,XbaI
PstI SalI
BamHI
XbaI,HindIII,SpII,PstI
BamHI,SalI,PstI
EcoRI,KpnI,NotI,KpnI,
EcoRI
SacI
PstI,SalI,BamHI,XbaI,
SmaI
HindIII,SpII,PstI
KpnI,SstI,SalI,
PstI
BamHI,XbaI,EcoRI,
ClaI,NcoI
PstI SalI
KpnI,NotI,HindIII
PstI,SalI,BamHI,XbaI
2678
456
567
556
300
1581
1000
1000
1000
300
1576
567
400
300
2600
1000
CaMV
35S
Adhi
PAT
sou
UBQp
UBQi
Alc R
sou
Alc A
Adhi
UBQi
UBQp
AMP
nos
CPT2-EPSPS
nos
CTP1-GOX
pMV2
16.18 kb

Fig.17.

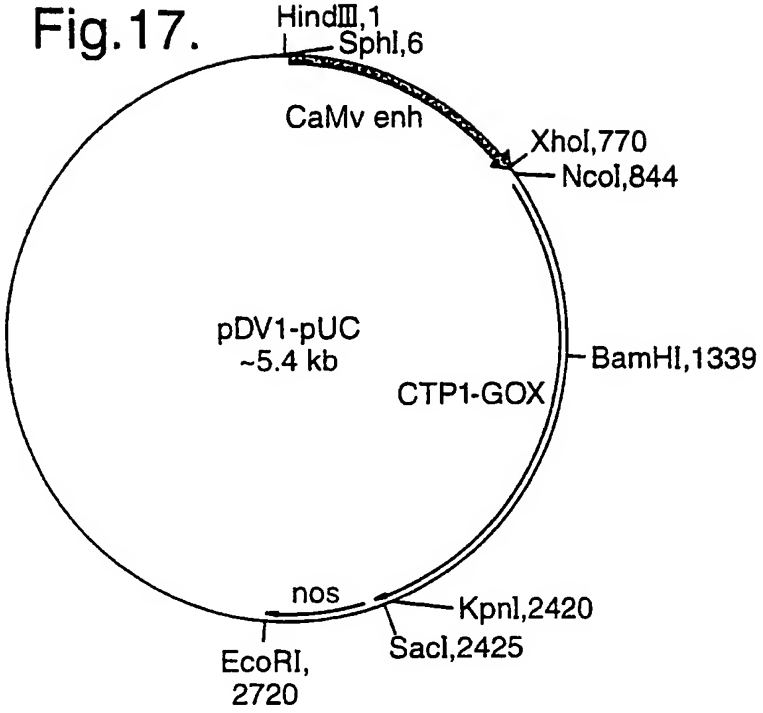

HindIII,1
SphI,6
CaMv enh
XhoI,770
NcoI,844
pDV1-pUC
~5.4 kb
BamHI,1339
CTP1-GOX
nos
KpnI,2420
SacI,2425
EcoRI,
2720

Fig.18.

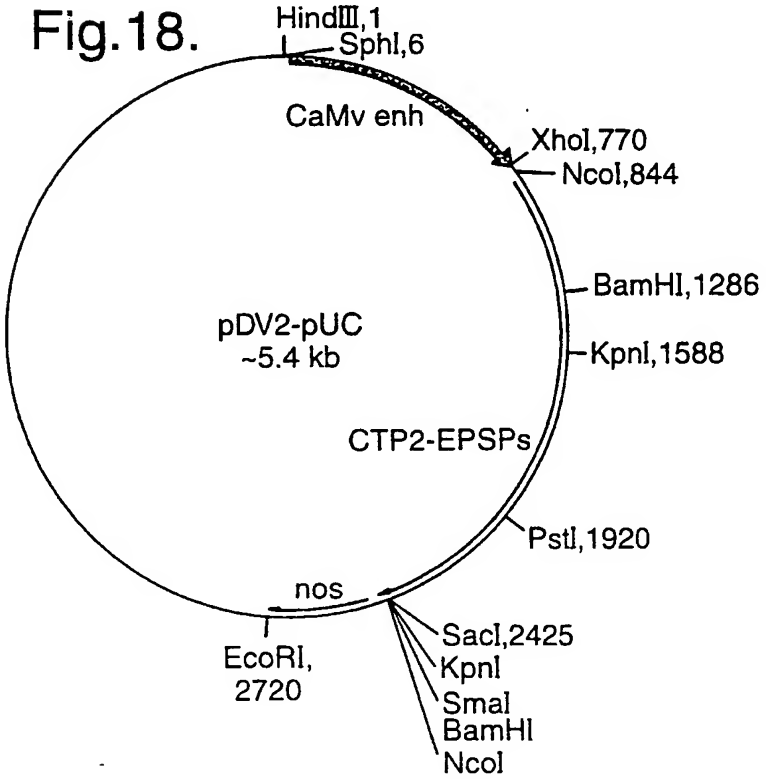

HindIII,1
SphI,6
CaMv enh
XhoI,770
NcoI,844
BamHI,1286
pDV2-pUC
~5.4 kb
KpnI,1588
CTP2-EPSPs
PstI,1920
nos
SacI,2425
KpnI
SmaI
BamHI
NcoI
EcoRI,
2720

Fig.19.

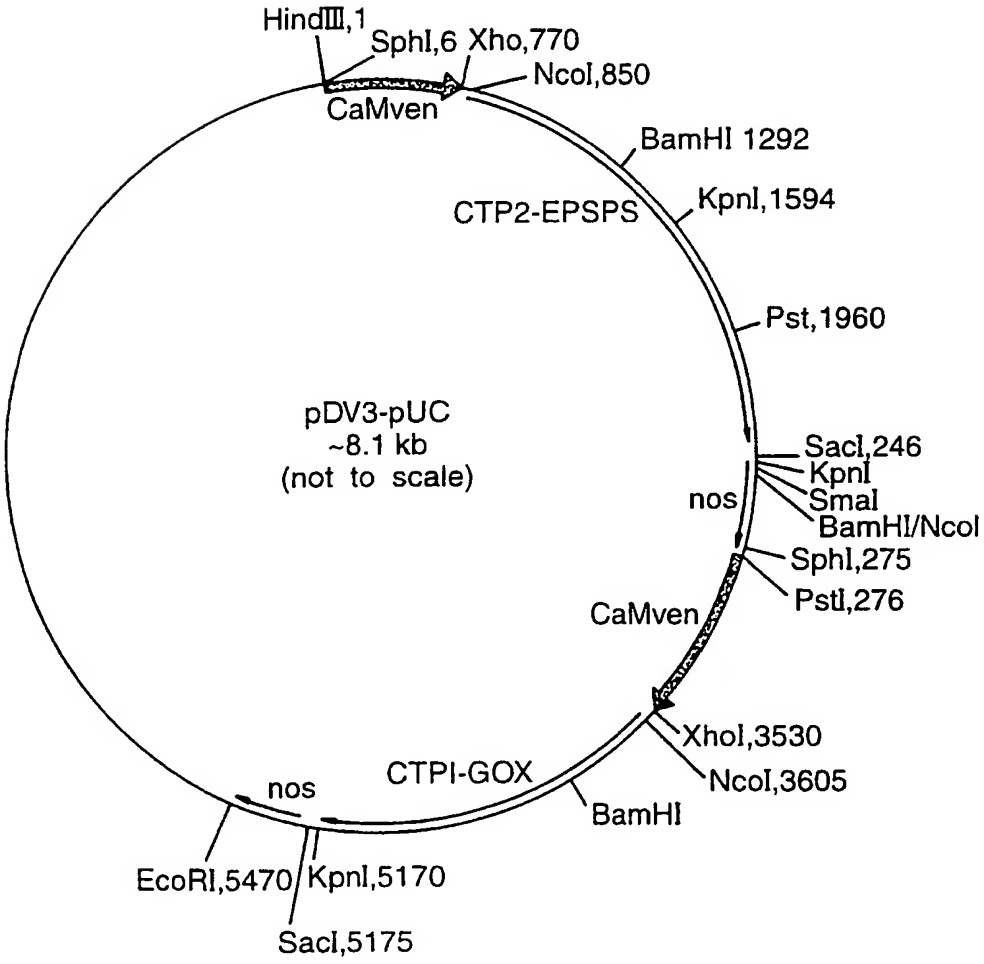

HindIII,1
SphI,6
Xho,770
NcoI,850
CaMven
BamHI 1292
CTP2-EPSPS
KpnI,1594
PstI,1960
pDV3-pUC
~8.1 kb
(not to scale)
SacI,246
KpnI
SmaI
BamHI/NcoI
nos
SphI,275
PstI,276
CaMven
XhoI,3530
NcoI,3605
CTPI-GOX
BamHI
nos
EcoRI,5470
KpnI,5170
SacI,5175

Fig.20.

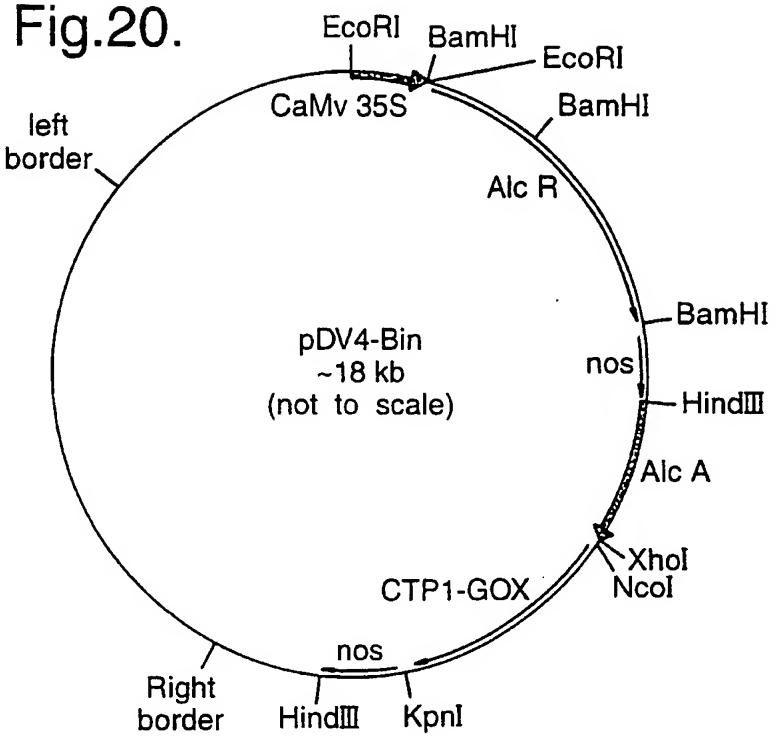

EcoRI
BamHI
EcoRI
CaMv 35S
BamHI
left
border
Alc R
BamHI
pDV4-Bin
~18 kb
(not to scale)
nos
HindIII
Alc A
XhoI
NcoI
CTP1-GOX
nos
Right
border
HindIII
KpnI

Fig.21.

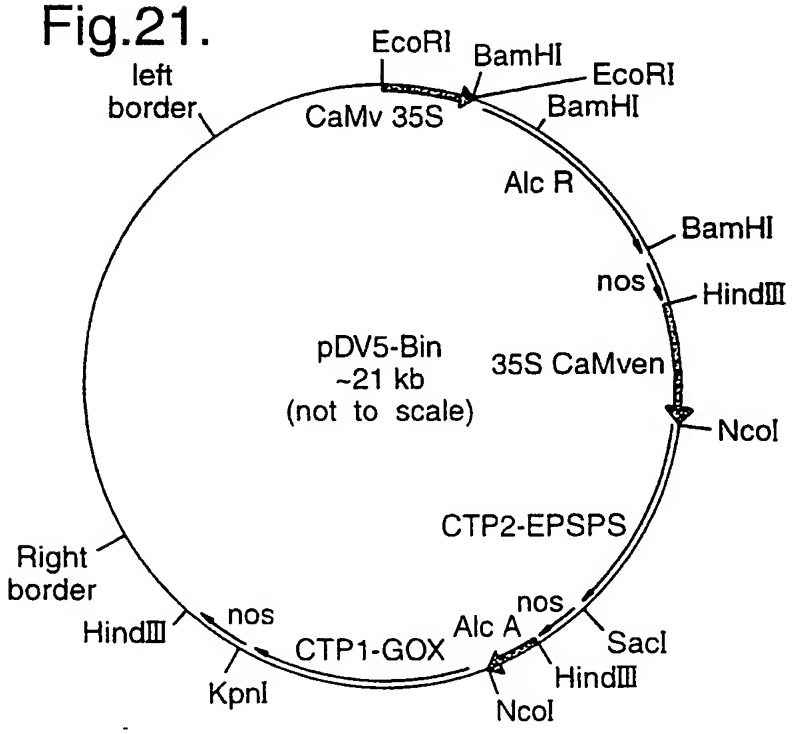
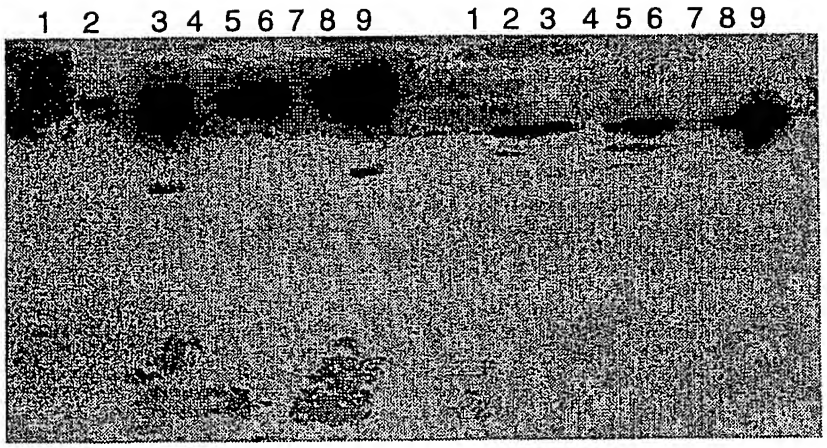

EcoRI
BamHI
left
border
EcoRI
CaMv 35S
BamHI
Alc R
BamHI
nos
HindIII
pDV5-Bin
~21 kb
(not to scale)
35S CaMven
NcoI
CTP2-EPSPS
Right
border
nos
HindIII
nos
Alc A
SacI
CTP1-GOX
HindIII
KpnI
NcoI

Fig.22.



INTERNATIONAL SEARCH REPORT

f national Application No
PCT/GB 96/01883

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 388 186 (DU PONT) 19 September 1990	1,3, 7-12,14, 15
Y	see page 10, line 35 - line 41 see page 78, line 1 - page 82, line 40 ---	3
Y	WO,A,93 01294 (ICI PLC) 21 January 1993 see page 11, line 16 - page 12, line 2 ---	3
X	WO,A,93 05164 (UNIV LEICESTER) 18 March 1993 see page 54, line 10 - line 18 see page 48, line 1 - line 19 --- -/--	1,3,8-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1997	29. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/01883

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 332 104 (CIBA-GEIGY) 13 September 1989 see page 17, line 25 - line 29 see page 22, line 41 - line 44 see page 36, line 55 - page 38, line 27 see page 58, line 30 - page 59, line 16 see page 40, line 50 - page 41, line 40	1,3, 8-12,14, 15
X	BIOTECHNOLOGY, vol. 5, no. 7, July 1987, pages 726-730, XP002022728 FILLATI, J.J., ET AL.: "Efficient transfer of a glyphosate tolerance gene into tomato using a binary agrobacterium tumefaciens vector" see page 729, right-hand column	1,2,7-14
A	WO,A,90 13658 (UNIV ALBERTA ;SZALAY ALADAR A (CA); LANGRIDGE WILLIAM A R (CA)) 15 November 1990 see the whole document	7
A	WO,A,93 21334 (ZENECA LTD ;CADDICK MARK XAVIER (GB); GREENLAND ANDREW JAMES (GB);) 28 October 1993 see the whole document	1-15
A	WO,A,92 00377 (MONSANTO CO) 9 January 1992 see the whole document	1-15
E	WO,A,96 25505 (PIONEER HI BRED INT) 22 August 1996 see examples 4,5	1,3,8-13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/01883

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0388186	19-09-90	AU-B-	638448	01-07-93
		AU-A-	5285490	22-10-90
		EP-A-	0463056	02-01-92
		JP-T-	4504054	23-07-92
		WO-A-	9011361	04-10-90
		US-A-	5364780	15-11-94
WO-A-9301294	21-01-93	AU-B-	672362	03-10-96
		AU-A-	2195992	11-02-93
		AU-A-	6210496	21-11-96
		CA-A-	2111983	21-01-93
		EP-A-	0603190	29-06-94
		JP-T-	6511385	22-12-94
		US-A-	5589614	31-12-96
WO-A-9305164	18-03-93	AU-B-	665778	18-01-96
		AU-A-	2485292	05-04-93
		CA-A-	2116775	18-03-93
		EP-A-	0602113	22-06-94
		JP-T-	6510198	17-11-94
EP-A-332104	13-09-89	AU-A-	3108089	14-09-89
		IL-A-	89495	31-08-95
		JP-A-	2009377	12-01-90
		PL-B-	162317	30-09-93
		PT-B-	89915	31-10-94
WO-A-9013658	15-11-90	NONE		
WO-A-9321334	28-10-93	AU-A-	3901993	18-11-93
		EP-A-	0637339	08-02-95
WO-A-9200377	09-01-92	AU-B-	655197	08-12-94
		AU-A-	8408591	23-01-92
		CA-A-	2083948	26-12-91
		EP-A-	0536330	14-04-93
		US-A-	5463175	31-10-95
WO-A-9625505	22-08-96	AU-A-	4924596	04-09-96